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Ana Margarida Ferreira Guimarães Pedrosa

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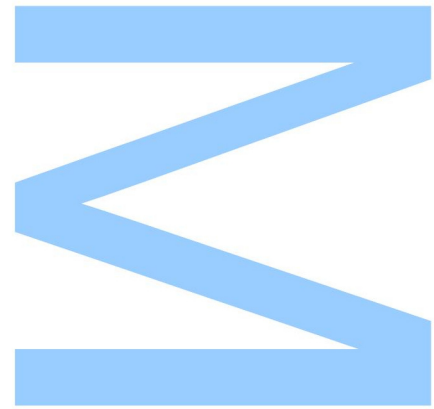

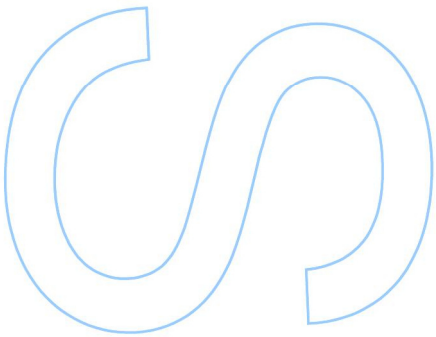

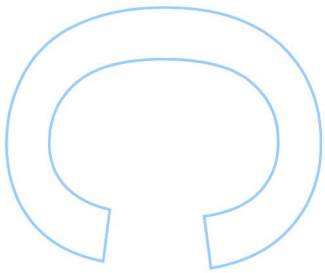

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RESUMO

A peroxina 5 (PEX5) é um recetor vaivém responsável pelo transporte de proteínas sintetizadas de novo destinadas para a matriz do peroxissoma. Tem uma estrutura modular caracterizada por i) um domínio N-terminal pobre em estrutura secundária/terciária que contém repetições de pentapéptidos aromáticos e uma cisteína evolucionariamente conservada, e ii) um domínio C-terminal conservado e estruturado constituído por várias repetições tetratricopeptídicas (TPRs). Depois de interagir com proteínas detentoras de uma sequência sinal de direcionamento para o peroxissoma ("peroxisomal targeting signal"; PTS), a PEX5 interage com o chamado "docking/translocation module" (DTM). Esta interação é dependente dos motivos aromáticos existentes na PEX5, e resulta, em última instância, na translocação da proteína-cliente para a matriz do organelo. No DTM, a PEX5 é monoubiquitinada na cisteína, e posteriormente extraída para o citosol pelo chamado "receptor export module" (REM). Estes dois últimos passos são ambos dependentes de ATP. A via de importação proteica mediada pela PEX5 é, obviamente, regulada. Presentemente, acredita-se que o mecanismo regulatório reside na própria PEX5. De facto, existe alguma evidência experimental sugerindo que a metade N-terminal da PEX5 (onde residem os domínios de interação com o DTM) interage com a parte C-terminal da PEX5, e que a interação de uma proteína-cliente com a região C-terminal da PEX5 destrói esta interação intramolecular ativando o seu domínio N-terminal. De acordo com este modelo, foi mostrado que uma PEX5 truncada (sem o domínio dos TPRs) é constitutivamente ativa, inserindo-se no DTM num processo que é independente de proteínas-cliente.

O objetivo deste trabalho foi adquirir mais informação sobre a estrutura/função da metade N-terminal da PEX5. Utilizando um sistema de importação/exportação *in vitro,* conseguimos demonstrar que um domínio compreendendo apenas os aminoácidos 1 a 117 da PEX5 tem a capacidade de interagir produtivamente com o DTM, ser monoubiquitinado e exportado para o citosol, num processo ATP-dependente, pelo REM. De igual forma, a remoção dos 9 resíduos iniciais da PEX5, que são conservados em várias espécies, resulta numa molécula perfeitamente funcional nos passos de "docking", inserção, ubiquitinação e exportação. Apesar deste domínio da PEX5 apresentar funcionalidade nos ensaios *in vitro*, moléculas de PEX5 que não possuam estes resíduos são ainda capazes de se inserirem no DTM de uma forma específica. De facto, os nossos dados mostram que uma PEX5 sem os primeiros 137 aminoácidos tem a capacidade de se inserir no DTM num passo que é dependente de proteínas-cliente. Tal observação sugere que o mecanismo auto-regulatório da PEX5 não reside nos seus primeiros 137 aminoácidos. Uma truncagem N-terminal na PEX5 ligeiramente maior,

i.e., uma PEX5 sem os primeiros 147 aminoácidos, resulta numa proteína incapaz de se inserir no DTM, sugerindo assim, que para moléculas de PEX5 que contenham a metade C-terminal, os aminoácidos 138 a 147 são essenciais para o passo de inserção.

ABSTRACT

Peroxin 5 (PEX5) is a shuttling receptor responsible for the transport of *de novo* synthesized proteins that are destined for the peroxisome matrix. PEX5 has a modular structure characterized by i) an N-terminal half with little secondary/tertiary structure that contains aromatic pentapeptide repeats and an evolutionary conserved cysteine and, ii) a conserved and structured C-terminal half composed by several tetratricopeptide repeats (TPRs). After interacting with proteins harbouring a peroxisomal targeting signal (PTS), PEX5 interacts with the so-called docking/translocation module (DTM). This interaction is dependent on the PEX5 aromatic motifs, and results, ultimately, in the translocation of cargo-proteins into the organelle matrix. Then, DTM-embedded PEX5 is monoubiquitinated at the cysteine, and extracted into the cytosol by the so-called receptor export module (REM). These last two steps are both dependent on ATP. The PEX5-mediated protein import pathway is, obviously, regulated. Presently, it is believed that the regulatory mechanism resides on PEX5 itself. In fact, there is some experimental evidence suggesting that the N-terminal half of PEX5 (where the DTM interaction motifs are located) interacts with its C-terminal half. Furthermore, the interaction of a cargo protein with the C-terminal region of PEX5 destroys this intramolecular interaction, therefore activating its N-terminal domain. In agreement with this model, it has been shown that a truncated PEX5 (without the TPRs domain) is constitutively active, being inserted into the DTM in a cargo-protein independent process.

The purpose of this work was to gather more information about the structure/function of the PEX5 N-terminal half. By resorting to an *in vitro* import/export system, we demonstrate that a domain harbouring just the PEX5 amino acid residues 1 to 117 has the capacity to productively interact with the DTM, to be monoubiquitinated and exported into the cytosol, in an ATP-dependent process, by the REM. Similarly, the removal of the initial 9 residues of PEX5, which are conserved among several species, results in a perfectly competent molecule in the docking, insertion, ubiquitination and export steps. Although this PEX5 N-terminal domain is functional in the *in vitro* assays, PEX5 molecules that do not contain these residues are still capable of being inserted into the DTM in a specific manner. In fact, our data shows that a PEX5 lacking the first 137 amino acid residues can be inserted into the DTM in a cargo-dependent process. This observation suggests that the PEX5 auto-regulatory mechanism does not reside in its first 137 amino acid residues. A slightly longer N-terminal PEX5 truncation, *i.e.*, a PEX5 without the first 147 amino acid residues, results in a protein incapable of being inserted into the DTM, thus suggesting that for PEX5 molecules containing the C-terminal half, amino acid residues 138 to 147 are essential for the insertion step.

KEYWORDS

PEX5, peroxisomal matrix protein import, monoubiquitination, pentapeptide motifs, docking/translocation module, receptor export module, peroxisomal targeting signal.

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LIST OF ABBREVIATIONS

^{35}S-	Radiolabelled protein at methionine residues with the ^{35}Sulphur radioisotope
AAA ATPases	ATPases associated with various cellular activities
AMP-PNP	Adenylyl-imidodiphosphate
ATP	Adenosine triphosphate
ATPγS	Adenosine 5'-*O*-(3-thiotriphosphate)
cSENP2	Catalytic domain of SENP2, a SUMO-protease
C-terminal	Carboxyl-terminal
Cys	Cysteine
DTM	Docking/translocation module
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligase enzyme
EDTA	Ethylenediamine tetraacetic acid
GSH	Reduced glutathione
GST-Ub	Fusion protein of glutathione-S-transferase and ubiquitin
HA-Ubal	Fusion protein of human influenza hemagglutinin (HA) and ubiquitin-aldehyde (Ubal)
HA-Ub-VME	Fusion protein of human influenza hemagglutinin (HA) and ubiquitin-vinyl-methyl-ester (Ub-VME)
His_6	Hexahistidine tag
Hs	*Homo sapiens*
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVT	*In vitro* Transcriptiontranscription/translation
K_D	Dissociation constant
MOPS	4-Morpholinepropanesulfonic acid
N-terminal	Amino-terminal
P	Organelle pellet obtained after PNS centrifugation
PBDs	Peroxisomal biogenesis disorders
PCR	Polymerase chain reaction
PEDs	Peroxisomal single enzyme/transporter deficiencies
PEX	Peroxisomal biogenesis factor gene
PEX	Peroxins or peroxisomal biogenesis factors
PEX5L	Long isoform of PEX5
PIM	Peroxisomal matrix protein import machinery

PK	Proteinase K
PMPs	Peroxisomal membrane proteins
PNS	Post-nuclear supernatant
PTS1	Peroxisomal targeting signal type 1
PTS2	Peroxisomal targeting signal type 2
RCDP	Rhizomelic chondrodysplasia punctata
REM	Receptor export module
Rpm	Rotations per minute
S	Supernatant fraction (*i.e.*, cytosol) obtained after PNS centrifugation
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
SUMO3	Small ubiquitin-like modifier 3
TBE	Tris-borate-EDTA
TCA	Trichloroacetic acid
TEV	Tobacco etch virus
TPRs	Tetratricopeptide repeats
Tris	Tris(hydroxymethyl)aminomethane
Ub	Ubiquitin
Ub-PEX5	Monoubiquitinated-PEX5
UCC	Ubiquitin-conjugating cascade
USP9X	Ubiquitin-specific protease 9X
WD-40	Protein motif of approximately 40 amino acid residues containing repeats of the tryptophan(W)-aspartate(D) dipeptide
WxxxF/Y	Di-aromatic pentapeptide repeat containing tryptophan (W) and either phenylalanine (F) or tyrosine (Y)
ZSS	Zellweger syndrome spectrum

1. INTRODUCTION

1.1 The Peroxisome

In 1966, de Duve and Baudhuin subjected a rat liver homogenate to a sucrose gradient centrifugation and identified microbodies containing high amounts of catalase and oxidative enzymes [1]. These cellular entities were termed “peroxisomes” for their production and detoxification of hydrogen peroxide. They are characterized by a globular shape with 0.1 to 1 μm in diameter and display a dense granular matrix occasionally containing a dense crystalloid core when analysed by electron microscopy (see Fig. 1) [1,2].

Peroxisomes are found in nearly all eukaryotic cells. Their number, size and function may vary according to the organism, cell type and both internal and exogenous signals [3]. There even are some specialized types of peroxisomes that receive other names, for instance, fungal Woronin-bodies, which protect against cell leakage after hyphal damage [4], plant glyoxysomes, which are responsible for carbohydrate anabolism [5], and trypanosomatid glycosomes that perform glycolysis [6].

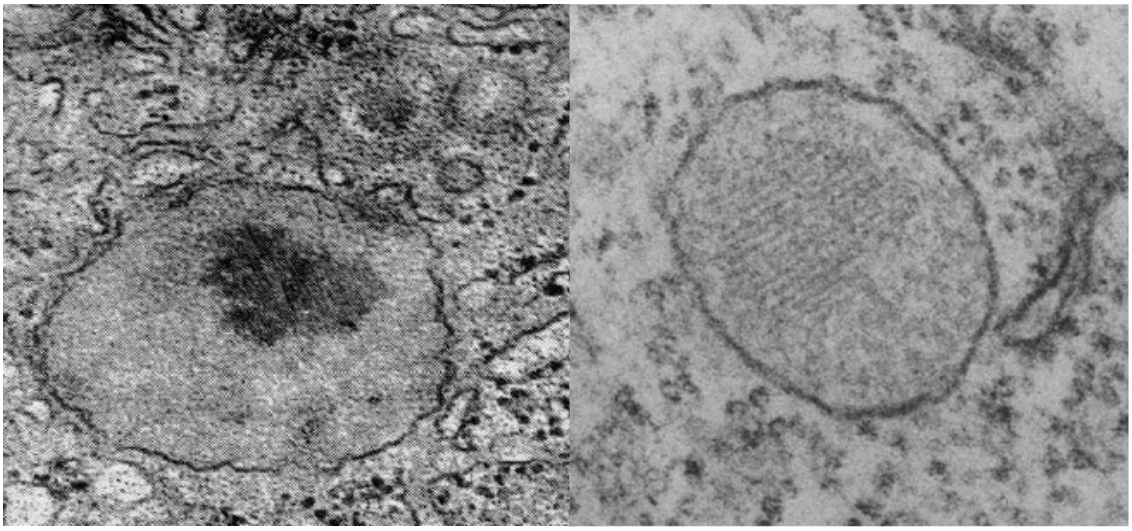

Fig. 1 - Electron micrographs of peroxisomes.
Left - Longitudinal section of a rat hepatocyte showing one single-membrane bound organelle with a granular matrix and a central core. From [1], (X 75,000).
Right - *Trypanosoma brucei* glycosome displaying one lipid bilayer and a dense centre, from [6], (X 172,000).

The peroxisomal matrix harbours enzymes responsible for more than 50 activities. In humans, these activities include the elimination of reactive oxygen species, the biosynthesis of ether-phospholipids and bile acids, the β-oxidation of very long chain fatty acids (VLCFA) and fatty acid α-oxidation [7].

1.2 Peroxisomes and disease

The correlation between peroxisomes and human disease was described in 1973 when it was reported that cells from Zellweger patients did not contain visible peroxisomal structures [8]. Presently, many peroxisome-associated diseases are known.

Together, they have an incidence of approximately 1:5000 individuals and are generally classified into two groups: the peroxisomal single enzyme/transporter deficiencies (PEDs) and the peroxisomal biogenesis disorders (PBDs) (reviewed in [9]). PEDs are caused by mutations in genes coding for peroxisomal matrix enzymes or membrane-embedded transporters and affect one or a few peroxisomal metabolic pathways. PBDs are the result of mutations in the so-called *PEX* genes encoding peroxins (or PEX proteins), which are proteins essential for peroxisomal biogenesis and inheritance. In the most severe cases of PBDs, all peroxisomal metabolic pathways are impaired.

1.2.1 Peroxisomal biogenesis disorders (PBDs)

PBDs are a disparate group of autosomal recessive syndromes that are subdivided into two categories: the Zellweger syndrome spectrum (ZSS) and the rhizomelic chondrodysplasia punctata (RCDP type 1 and type 5) [9].

Nowadays, the ZSS comprises four disorders that represent a severity spectrum. In an order of decreasing severity, these are: 1) the cerebrohepatorenal syndrome (Zellweger disease); 2) the neonatal adrenoleukodystrophy (NALD); 3) the infantile Refsum disease (IRD) and 4) the Heimler syndrome (HS) [9]. Clinical manifestations of the ZSS include hypotonia, hepatic and/or renal dysfunction, developmental delay, craniofacial malformations and seizures [9]. These clinical manifestations are accompanied by metabolic alterations such as the accumulation of VLCFA and the absence of plasmalogens. At the cell level, these patients may have few or even no peroxisomes, or they may contain peroxisomal ghosts (these are organelles deprived of matrix proteins) [10]. The Heimler syndrome (HS) is probably the mildest PBD. It is due to alterations in *PEX1* and *PEX6* genes and is characterized by hearing loss and abnormalities in teeth's enamel and nails [11]. Overall, the Zellweger spectrum is associated with mutations in 13 *PEX* genes. These are *PEX1*, *PEX2, PEX3, PEX5, PEX6, PEX10, PEX11β, PEX12, PEX13, PEX14, PEX16, PEX19 and PEX26* (see also Table 2) [9,10].

RCDP shares with ZSS several clinical presentations but reduction of the limbs and punctate epiphyseal calcifications are unique to RCDP [9]. RCDP type 1 is due to alterations in the gene encoding the peroxin PEX7 [12], which is responsible for the import of a small subset of peroxisomal matrix proteins necessary for plasmalogen synthesis and the metabolism of phytanic acid [9]. RCDP type 5 was identified in only a few patients and arises from a mutation in *PEX5* that specifically affects the import of proteins recognized by the PEX7 co-receptor (for more details see section 1.3.2.1 and 1.4) [13].

1.2.2 Peroxisomal single enzyme/transporter deficiencies (PEDs)

Given that modifications in individual peroxisomal enzymes or transporters lead to the impairment of the metabolic pathway where they intervene, PEDs may be classified according to the functions affected [14]. The clinical and biochemical phenotypes of PEDs are more dispersed. A list of the presently known deficiencies is presented in Table 1 [9].

Table 1 - List of peroxisomal single enzyme deficiencies.
PEDs are listed according to the peroxisomal pathway affected, the modified protein and the gene involved. Adapted from [9,15].

Impaired peroxisomal pathway	Disease	Enzyme/Transporter involved	Gene
Fatty acid β-oxidation	X-linked adrenoleukodystrophy (X-ALD)	ALDP	*ABCD1*
	Acyl-CoA oxidase 1 deficiency	ACOX1	*ACOX1*
	Acyl-CoA oxidase 2 deficiency	ACOX2	*ACOX2*
	D-bifunctional protein deficiency	DBP	*HSD17B4*
	Sterol Carrier Protein X deficiency	SCPx	*SCP2*
	2-Methylacyl-CoA racemase deficiency	AMACR	*AMACR*
Fatty acid α-oxidation	Refsum disease	Phytanoyl-CoA 2-hydroxylase	*PHYH*
Glyoxylate metabolism	Primary Hyperoxaluria type 1	Alanine:glyoxylate aminotransferase (AGT)	*AGXT*
Ether phospholipid biosynthesis	RCDP type 2	Dihydroxyacetonephosphate acyltransferase (DHAPAT)	*GNPAT*
	RCDP type 3	Alkyl-dihydroxyacetonephosphate synthase (ADHAPS)	*AGPS*
	RCDP type 4	Fatty acyl-CoA reductase 1 (FAR1)	*FAR1*
Bile acid synthesis	ABCD3 deficiency	PMP70	*ABCD3*
	BAAT deficiency	Bile acid-CoA:amino acid N-acyltransferase	*BAAT*
Hydrogen peroxide metabolism	Acatalasemia	Peroxisomal catalase	*CAT*

1.3 Peroxisome Biogenesis

Currently, 14 mammalian *PEX* genes have been implicated in the biogenesis and inheritance of peroxisomes [13,16]. Peroxisomes lack genetic information and so their proteins are encoded on the nuclear DNA, synthesized on the cytosol and delivered to their final peroxisomal destination (the organelle membrane or its matrix) [2,17].

The biogenesis of the peroxisomal membrane is a controversial issue among the scientific community and two models have been proposed: 1) "*de novo* formation

pathway" and 2) the "growth and division model". The latter proposes that the organelles grow and then undergo fission yielding daughter organelles that are replenished with new peroxisomal membrane and matrix proteins [2,18,19]. The *de novo* formation pathway is based on the finding that lack of PEX3, PEX16 or PEX19 results in a seemingly complete absence of peroxisomes; the reintroduction of the missing component leads to the appearance of fully functional peroxisomes, apparently formed *de novo* (reviewed in [16]).

Regardless of these controversies, it is obvious that from a conceptual point of view, biogenesis of an organelle requires 1) formation of the lipid bilayer, 2) insertion of peroxisomal membrane proteins (PMPs) into the organelle membrane and, 3) import of peroxisomal matrix proteins [16].

1.3.1 Peroxisomal membrane assembly

Peroxisomes do not contain most of the enzymes required for membrane lipid biosynthesis. Therefore, they receive them from other sources such as the endoplasmic reticulum (ER) [20,21]. In support of this idea, contact sites between these two subcellular compartments have been observed by electron microscopy and transfer of lipids between them has been documented in yeast [22–24].

The machinery involved in this lipid transport process remains largely unknown. Nevertheless, remodelling of the membrane seems to involve PEX11, which has been shown to induce elongation and tubulation of peroxisomes, a process probably coupled to the incorporation of new lipids into the peroxisomal membrane [25–29]. In addition to lipids, it is obvious that the peroxisomal membrane needs also to be populated with proteins to yield a mature and fully functional organelle. How these proteins are targeted and inserted into the organelle membrane is still not fully comprehended. Yet, it is clear that two integral peroxisomal membrane proteins, PEX3 and PEX16, plus the cytosolic chaperone PEX19 are required (reviewed in [16]). According to current models, PEX19 binds newly synthesized PMPs in the cytosol and delivers them to the membrane embedded-PEX3 protein [30–32]. Somehow these interactions culminate with the insertion of the PMPs into the peroxisomal membrane and in the release of PEX19 back to the cytosol [31,32]. In the specific case of PEX3 and probably also PEX16, the *de novo* synthesis model postulates a different pathway. Indeed, according to some authors these proteins are co-translationally inserted into the ER, from where they are then directed to the peroxisome [29,33].

1.3.2 The peroxisomal matrix protein import pathway

Import of peroxisomal matrix proteins requires a unique but extremely conserved machinery (see Table 2), the so-called peroxisomal matrix protein import machinery (PIM) [34–36]. Impressively, this machinery is capable of internalizing folded and even oligomeric proteins [37–39]. Many different strategies have been used to dissect the mechanism of the PIM, from *in vitro* and *in vivo* functional studies to the isolation and analysis of protein complexes. Collectively, the data gathered by many different laboratories suggest that this import pathway involves 3 phases: 1) the recognition of a cytosolic cargo protein by the shuttling receptor; 2) the docking and insertion of receptor-cargo protein complex into the peroxisomal membrane with concomitant cargo release into the organelle lumen; and, 3) the recycling of the shuttling receptor back to the cytosol. Details on of each of these steps are provided below.

Table 2 - Proteins involved in the peroxisomal matrix protein import pathway, their subcellular localization and function.
Adapted from [40–42]. Abbreviations: Cyt, cytosol; Membr, membrane; M, mammals; P, plants; F, fungi; Y, yeast; PTS1, peroxisomal targeting signal 1; PTS2, peroxisomal targeting signal 2; DTM, docking/translocation module; REM, receptor export module; Ub-PEX5, monoubiquitinated PEX5; *, proteins associated with other cellular functions and not exclusive of the peroxisome biogenesis.

Protein	Localization	Organism	Purpose
PEX5	Cyt/Membr	M, P, F, Y	Receptor for PTS1 protein Receptor for PTS2 proteins in M & P
PEX7	Cyt/Membr	M, P, F, Y	Co-receptor for PTS2 proteins
PEX13	Membr	M, P, F, Y	Transmembrane DTM component
PEX14	Membr	M, P, F, Y	Transmembrane DTM component
PEX2	Membr	M, P, F, Y	E3 ubiquitin-ligase
PEX10	Membr	M, P, F, Y	E3 ubiquitin-ligase
PEX12	Membr	M, P, F, Y	E3 ubiquitin-ligase
PEX1	Cyt/Membr	M, P, F, Y	Receptor export
PEX6	Cyt/Membr	M, P, F, Y	Receptor export
PEX26	Membr	M, F, Y	REM membrane anchor
E1*	Cyt	M, P, F, Y	Ubiquitin-activating enzyme
E2D1/2/3*	Cyt	M	E2 ubiquitin-conjugating enzyme
AWP1*	Cyt	M	Adaptor protein that probably mediates the interaction of the REM with Ub-PEX5
USP9X*	Cyt	M	Desubiquitinase

1.3.2.1 The peroxisomal targeting signals and their receptors

As previously mentioned, peroxisomal matrix proteins are translated on free cytosolic ribosomes and post-translationally transported to their final peroxisomal destination [2,34]. In order to be delivered to the peroxisome lumen, these proteins must display a peroxisomal targeting signal (PTS) which can be of two types. The majority contain a carboxyl(C)-terminal PTS-type 1 (PTS1), which comprises a tripeptide with the consensus sequence (S/A/C)-(K/H/R)-(L/M) [43,44]. A small number of matrix proteins

contain instead a PTS-type 2 (PTS2) [45]. This is a degenerated amino(N)-terminal nonapeptide characterized by the consensus sequence R-(L/V/I/Q)*xx*(L/V/I/H)(L/S/G/A)*x*(H/Q)(L/A) [46,47]. In contrast to the PTS1, the PTS2 is generally cleaved after import [48–52]. In mammals, both PTS1 and PTS2 proteins are transported to the peroxisome by the shuttling receptor PEX5 [53–57]. For PTS1-containg cargoes no protein other than PEX5 is necessary for this process [58–62]. For PTS2-proteins, PEX5 needs the help of PEX7, a WD-40 repeat protein which interacts both with PEX5 and the PTS2 (see also section 1.4) [49,53,54,56,57,63].

1.3.2.2 Docking and translocation of receptor-cargo proteins

After binding a cargo-protein in the cytosol, the receptor PEX5 interacts with a peroxisomal membrane protein complex - the so-called docking/translocation module (DTM) - through several conserved motifs present in the PEX5 N-terminal half (see section 1.4 and Fig. 2, 1b) [64–69]. The mammalian DTM is composed by PEX14 and PEX13, plus a complex of E3 ubiquitin-ligases comprising PEX2, PEX10 and PEX12, each containing a RING(really-interesting-new-gene)-Zn^{2+} binding domain [70–72]. The interaction between the complex PEX5-cargo protein and the DTM, occurs in two steps: 1) docking, and 2) insertion. The first can occur at low temperatures (0 ºC) and is completely reversible (PEX5 at this step is referred to as stage 1b PEX5; see Fig. 2); the second, on the contrary, is irreversible in the absence of cytosolic ATP and requires higher temperatures [34,73,74]. Strikingly, after the insertion step, PEX5 displays a transmembrane topology exposing a small N-terminal segment (of *ca.* 2-kDa) into the cytosol, whereas the remaining polypeptide chain faces the matrix side of the membrane. This is the so-called stage 2 PEX5 (see Fig. 2) [75–77]. Importantly, experiments using an *in vitro* system, which recapitulates all the steps of the PEX5-mediated protein import pathway, have shown that insertion of PEX5 into the DTM is cargo-dependent and ATP-independent [75,76,78]. These and other findings led to the proposal that the driving force for protein translocation across the peroxisomal membrane resides in the strong protein-protein interactions that are established between PEX5, on one side, and components of the DTM on the other (*e.g.,* PEX14 and PEX13) [34,78,79]. Insertion of the PEX5-cargo protein complex into the DTM culminates with the release of the cargo-protein into the organelle matrix [74,80]. Considering that, in at least some cases, the PEX5-cargo protein interaction is very strong (which translates into very high half-lives of these complexes) [76,81]; it has been proposed that cargo release is triggered by DTM-induced conformational alterations in the receptor [81,82].

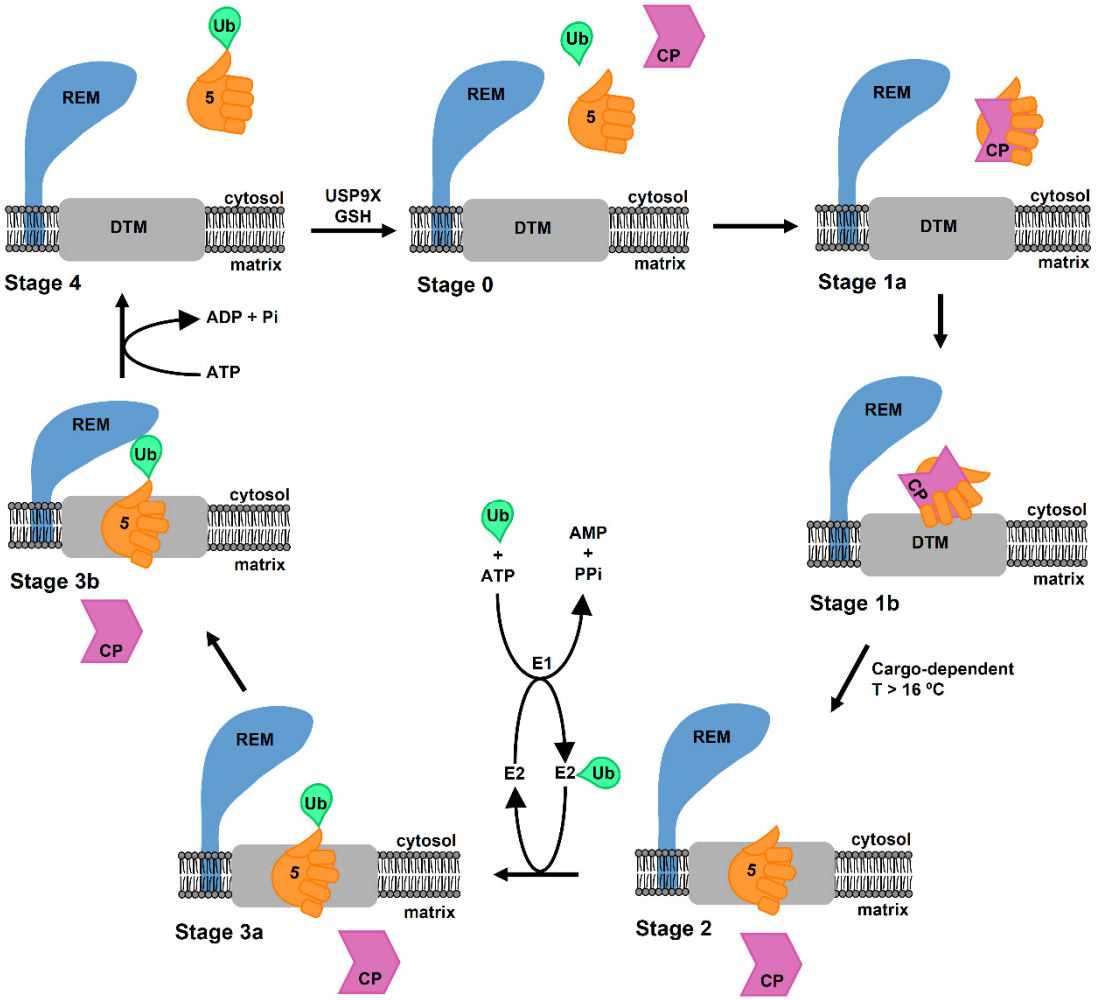

Fig. 2 - The mammalian peroxisomal matrix protein import cycle.
Schematic representation of the PEX5-mediated peroxisomal protein import, please note that sub-stages "a" and "b" are theoretical. For simplicity, PEX7 is not represented. Nevertheless, PEX7 binds cytosolic PTS2-proteins and is escorted by PEX5 through the displayed stages of this pathway. **Stage 0** - The cytosol contains free PEX5 (5, orange, hand-shaped), ubiquitin (Ub, green) and newly synthesized cargo proteins (CP, pink). **Stage 1a** - PEX5 recognizes and binds a cargo protein destined for the peroxisomal lumen. **Stage 1b** - The receptor-cargo complex docks at the DTM peroxins (DTM, grey, rectangle). **Stage 2** - The PEX5-cargo protein complex gets inserted into the DTM in a cargo- and temperature-dependent manner (T>16 ºC). Here, the establishment of strong protein-protein interactions between PEX5 and the DTM components is suggested to drive translocation and release of the cargo protein into the peroxisomal matrix. **Stage 3a** - Membrane-embedded PEX5 is monoubiquitinated at Cys11 in a process dependent on ATP. **Stage 3b** - Quickly, Ub-PEX5 is recognized by the REM (blue complex). **Stage 4** - The REM couples ATP hydrolysis to the removal of Ub-PEX5 from peroxisomal membranes. Once again in the cytosol, Ub-PEX5 is deubiquitinated by enzymatic (USP9X) and non-enzymatic mechanisms (GSH) yielding a free receptor optimal for further import rounds -**Stage 0**.

1.3.2.3 Monoubiquitination and recycling of the shuttling receptors

As mentioned above, insertion of PEX5 into the DTM is an irreversible process [73,74]. Thus, it is not surprising that disruption of the PEX5-DTM interaction requires the chemical energy from ATP hydrolysis [78,83]. Remarkably, the ATP-dependent extraction of receptors back into the cytosol comprises two different steps: 1) ubiquitination of PEX5 at an evolutionary conserved cysteine present at its N-terminal and, 2) extraction of monoubiquitinated-PEX5 (Ub-PEX5) by a peroxisomal protein complex, the so-called receptor export module (REM), which comprises the two AAA

ATPases, PEX1 and PEX6, that are anchored to the peroxisomal membrane by the third subunit of the REM, PEX26 [73,84–87].

Several mechanistic details on each of these steps are already known. Monoubiquitination of PEX5 displays some unique properties. Indeed, in contrast to the vast majority of ubiquitination reactions occurring in the cell, which involves the formation of an isopeptide bond between the carboxyl-terminus of ubiquitin and the ε-amino-group of a lysine residue in the targeted proteins, here, the acceptor of ubiquitin is a conserved cysteine residue in PEX5 (Cys11 in human PEX5) [84,88,89]. The mammalian E2s involved in this conjugation have been identified [90]. These are the E2D1/2/3 ubiquitin-conjugating enzymes (also known as UbcH5a/b/c in humans). It is presently accepted that ubiquitin transthiolation from the E2 to PEX5 is catalysed by the RING-finger peroxins, PEX2, PEX10 and PEX12, but which of these three peroxins (if any alone) is specifically involved in this event remains completely unknown [55,72,91–93].

Monoubiquitination of PEX5 at the DTM is rapidly followed by its ATP-dependent extraction by the REM [78,84,85,89,94,95]. Although we are still lacking molecular details on this step, recent data suggest that the AAA ATPases may use a threading mechanism to remove Ub-PEX5 from the DTM. Indeed, it has been shown that a PEX5 molecule fused to a bulky, tightly folded, C-terminal tag is recognized by the REM but only partially extracted from the DTM. Seemingly, the REM cannot thread tightly folded domains [96].

Once Ub-PEX5 is in the cytosol, it is rapidly deubiquitinated. In fact, Ub-PEX5 was never detect in cytosolic fractions from rat liver, indicating that *in vivo* the deubiquitination step is much faster than the extraction of Ub-PEX5 from the DTM [89,97]. Data from our laboratory suggests that Ub-PEX5 can be deubiquitinated by two mechanisms [89,97]. One, is based on the lability of the thioester link between ubiquitin and PEX5, and postulates that small nucleophiles such as glutathione can disrupt this bond. The other, involves a deubiquitinase which in human and rat has been identified as USP9X [97].

The deubiquitination of PEX5 is the last step of this protein import pathway. The receptor can now bind another cargo-protein and promote its transport to the peroxisomal lumen [93].

1.4 Functional and structural relationships in PEX5

In mammals, the *PEX5* gene actually gives rise to two different PEX5 proteins, the so-called PEX5S (S as in short) and PEX5L (L as in long). The two isoforms originate from an mRNA alternative splicing mechanism [53]. Both these proteins are competent in transporting PTS1-proteins to peroxisomes, but only PEX5L can interact with PEX7

and thus, only this isoform of PEX5 can transport PTS2-proteins to the organelle [53,54,98].

Human PEX5L is a monomeric protein of 639 amino acids, which corresponds to a theoretical molecular mass of 70.9 kDa [99,100]. However, the protein migrates with a larger apparent molecular mass upon SDS-PAGE due to the fact that it contains very few hydrophobic amino acid residues in its polypeptide chain (see also below) [99,101].

Structurally, PEX5 comprises two main domains (see Fig. 3). Its C-terminal half contains seven tetratricopeptide repeats (TPRs) [58,61,62].This is the most well-conserved region of PEX5 among species [54,61,102–104]. The TPR repeats of PEX5 are organized into two clusters – TPR 1 to 3 and TPR 5 to 7 – with TPR4 functioning as a hinge that connects them [61,62]. It is this domain of PEX5 that binds the PTS1 signal. The C-terminal PTS1 peptide is bound in a pocket formed by the two TPRs clusters (see Fig. 3) [62]. Although this is probably the most important binding interface between PEX5 and PTS1 cargoes, it is surely not the only one. Indeed, a variety of studies have shown that the N-terminal half of PEX5, besides interacting with PEX7 and the PTS2 signal, also interacts directly with PTS1 cargo proteins [81,82,98,105–109].

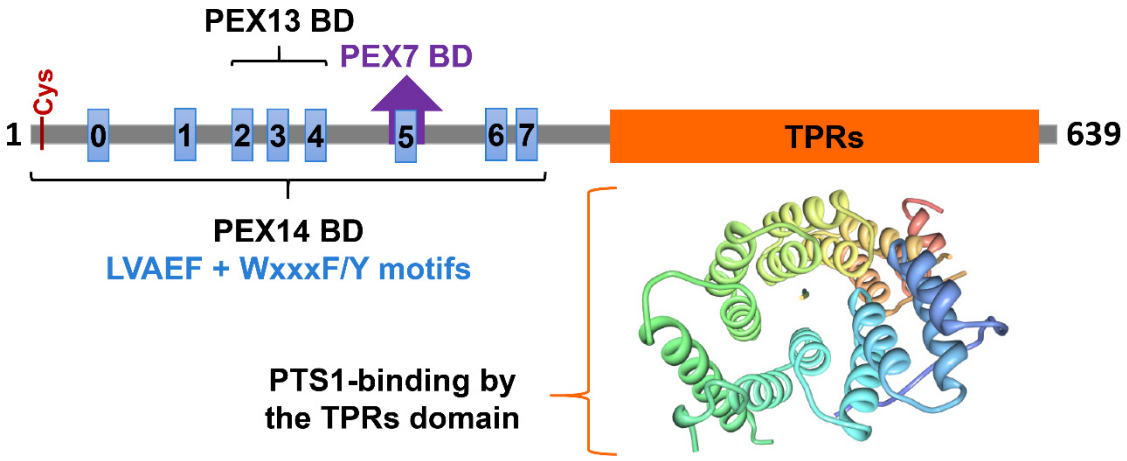

Fig. 3 - Functional components of the mammalian peroxisomal protein import receptor PEX5.
Scheme depicting the PEX5L bimodal configuration: a structured C-terminal (orange box "TPRs") and a natively unfolded N-terminal domain. At the N-terminal there are 8 pentapeptide motifs (7 WxxxF/Y, plus the motif LVAEF) responsible for the interaction of PEX5 with the DTM components, PEX13 and PEX14. The N-terminal domain of PEX5 also contains a PEX7 binding domain (in purple) which is crucial for the targeting of PEX7-PTS2 complexes to peroxisomes. The PTS1-signal is bound at the C-terminal of PEX5 by two clusters of tetratricopeptide repeats (TPRs 1-3 and 5-7) as shown in the three-dimensional (3D) representation of the PEX5-PTS1 complex along the PTS1-peptide axis [62]. "BD" stands for binding domain. The colours in the 3D representation of the TPRs domains range from blue to red which corresponds to the TPRs 1 to 7.

The second main domain of PEX5 comprises its N-terminal half. A variety of biochemical and biophysical experiments have shown that this is a "natively unfolded pre-molten globule-like domain" [101]. Proteins in this family can have some secondary structure but they lack a stable tertiary fold [110]. Natively unfolded proteins/domains display very interesting properties. For instance, many of these proteins have a higher

probability to encounter its binding partners because they can use a so-called “fly-casting” mechanism [111]. It is in this N-terminal domain of PEX5 that resides the evolutionary conserved cysteine, the residue that is ubiquitinated at the DTM as explained above [84]. It is also in this domain that seven conserved di-aromatic pentapeptide repeats with the sequence WxxxF/Y, as well as a less conserved LVxEF pentapeptide motif, are found [65,67,68,70,77,102,103,112–114]. We will refer to the latter as pentapeptide 0 and to the former as pentapeptides 1 to 7.

All these pentapeptides interact directly with PEX14 in *in vitro* binding assays, although *in vivo* only some of them seem to be crucial for peroxisomal matrix protein import [67]. The interaction site on PEX14 for these pentapeptides resides in the N-terminal domain of this protein (amino acids 1-78) [34,112–114]. The K_Ds for pentapeptide-PEX14 interactions have been determined (see Table 3) [113,114]. They are all in the low nanomolar range and thus, they might explain why insertion of PEX5 into the DTM is an irreversible event. In addition to PEX14, some of the pentapeptide motifs in PEX5 have also been implicated in the interaction with another DTM component, PEX13 [65,69,99,115]. According to Otera *et al.*, the region comprising pentapeptide motifs 3 and 4 interacts directly with this membrane peroxin [67].

Table 3 - Dissociation constants (K_D) for the interaction of the PEX5 pentapeptide motifs with PEX14(1-78).
Synthetic peptides harbouring the respective pentapeptide repeat (0 to 7) were incubated or not with PEX14-NTD (amino acid residues 1-78). The dissociation constants (K_D) for these protein complexes were determined by intrinsic tryptophan fluorescence measurements (ITF) [114] or by surface plasmon resonance (SPR) [113], the average of these measurements is presented. The sequence of the aromatic motifs and their position in PEX5 are displayed in the 2nd column. Note that the PEX5 synthetic peptides analysed correspond to broader PEX5 regions, referred in the 4th column.

Motif Nº	Sequence (amino acid position)	Average K_D for PEX14-NTD (nM)	PEX5 region analysed / technique
0	LVAEF (62 – 66)	157	1-110 / SPR
1	WAQEF (118 – 122)	7	113-128 / ITF
2	WSQEF (140 – 144)	9	135-150 / ITF
3	WAEEY (159 – 163)	17	154-169 / ITF
4	WYDEY (184 – 188)	109	179-194 / ITF
5	WAAEF (243 – 247)	8	238-252 / ITF
6	WVDQF (257 – 261)	29	252-267 / ITF
7	WLSDY (308 – 312)	35	303-318 / ITF

Although the PEX5-DTM interaction seems to be simply mediated by the small pentapeptide motifs on PEX5, insertion of this receptor into the DTM does not just happen by simple collision of the two molecular species. Actually, in the absence of cargo-proteins, virtually no PEX5 enters the DTM [75]. Strikingly, this cargo-dependent insertion of PEX5 into the DTM is only true for the full-length, fully functional protein; truncated PEX5 molecules lacking the C-terminal TPRs domain or a PEX5 containing a

single missense mutation (N526K) that abolishes its PTS1 binding activity, no longer display this property, *i.e.,* they are constitutively active in the insertion step [75,116]. These findings strongly suggest that insertion of PEX5 into the DTM is a regulated process and that the regulatory mechanism does not rely on the DTM (seemingly, it can accept PEX5 species lacking cargo proteins) but rather on PEX5 itself [75]. These data are at the basis of a model in which a PTS1-binding domain acts as a *cis*-acting repressor of the N-terminal half of PEX5, the region of the receptor that interacts with the DTM [75]. Such a model postulates the existence of a cross-talk between the two main domains of PEX5 but the precise regions involved in this intramolecular interaction remain completely unknown.

2. AIMS

Although our knowledge on the mechanism of the peroxisomal protein import machinery has increased dramatically in recent years, we are still far from comprehending many of its details. Since PEX5 is involved in all the steps of peroxisomal matrix import, characterizing its behaviour during this cycle is crucial to better understand this biological pathway. It is known that the C-terminal half of PEX5 is important for PTS1-cargo binding allowing PEX5 to be in an import-competent state. It is also known that the N-terminal half is important for: 1) PTS2-protein import, 2) interaction with the DTM and, 3) extraction of PEX5 from the DTM. This work is centred on the functional characterization of the N-terminal half of PEX5.

The PEX5 autoinhibition model suggests that there is an intramolecular interaction between the C-terminal and N-terminal half of cargo-free PEX5, which blocks the DTM-interacting motifs in the N-terminal of PEX5. Therefore, the first aim of this work was to define the minimum domain of PEX5 capable of binding PTS1-cargoes and entering the DTM in a specific process. For this purpose, we produced N-terminally truncated PEX5 molecules and studied their properties in *in vitro* import assays (note that these PEX5 proteins do not have cysteine 11 and therefore cannot be recycled back into the cytosol). Naturally, this task might also lead to the identification of a PEX5 truncated protein still capable of entering the DTM, but now in a cargo-independent manner. The identification of such a PEX5 molecule would provide valuable data on the auto-regulatory mechanism of PEX5.

In the second part of this work we wanted to delimit the minimum domain of PEX5 with the capacity to: 1) enter the DTM and be monoubiquitinated, and, 2) be exported by the REM. Thus, several short C-terminally truncated versions of PEX5 were produced and characterized using *in vitro* import assays. Our aim was to better define the binding interface(s) between PEX5 on one side, and the DTM and the REM on the other.

Finally, if the previous tasks led to the identification of two non-overlapping PEX5 proteins, both capable of entering the DTM but only one of them competent in the export step, then we could use these proteins to gather valuable mechanistic information on the action of the REM. Specifically, the demonstration that export of a truncated PEX5 molecule could drive the export of a *per se* export-incompetent PEX5 species, would suggest that the REM acts on the DTM itself.

3. EXPERIMENTAL PROCEDURES

3.1 *Homo sapiens* PEX5 DNA constructs

Truncated versions of PEX5 were constructed by polymerase chain reaction (PCR) (with either NZYProof DNA polymerase MB14601 or Invitrogen™ Platinum™ Pfx DNA Polymerase) using as template pGEM-4-PEX5L(C11K) and pGEM-4-PEX5L(C11A) [89]. Both plasmids encode full-length versions of *Homo sapiens* PEX5L containing the missense mutation that replaces cysteine 11 with a lysine (K) or an alanine (A) residue, respectively. In these plasmids, the gene of interest was cloned between SalI restriction sites which places a guanine both at position -3 and +4 relative to the AUG initiator codon, thus providing a stable variation of the Kozak sequence [117]. cDNAs corresponding to *Hs*-PEX5L(C11A) or *Hs*-PEX5L(C11K) amino acids residues 1 to 61, residues 1 to 61 plus three extra C-terminal methionines, residues 1 to 117, residues 1 to 125, residues 1 to 139, residues 1 to 158, residues 1 to 181 and residues 1 to 197 were obtained by PCR with the primers presented in Table 4. The same forward primer, which anneals at a pGEM-4 region located 67 base pairs upstream the T7 DNA polymerase promoter was used to produce all the cDNAs encoding PEX5 C-terminal truncations. The amplified DNA fragments were gel-purified (NzyGelPure MB01101), quantified by agarose electrophoresis and used in *in vitro* transcription/translation (IVT).

pGEM-4-PEX5(C11A)ΔC1 (described in [118]) codes for a protein comprising amino acids 1 to 324 of *Homo sapiens* PEX5 harbouring the mutation C11A. The plasmid pGEM-4-PEX5(C11K)ΔC1 (obtained by Doctor Cláudia Grou) encodes residues 1 to 324 of *Homo sapiens* PEX5 comprising the C11K mutation (unpublished). The plasmid pET28a-PEX5ΔN147 encodes residues 148 to 639 of PEX5 [82].

PEX5(C11K)10-324 is a protein that comprises amino acid residues 10 to 324 of *Homo sapiens* PEX5 with an upstream initiator methionine. Its cDNA was obtained by PCR using as a template pGEM4-PEX5L(C11K) (see primers in Table 4). The resultant PCR product was gel-purified and sequentially digested with XbaI (Fermentas) and SalI (Takara), followed by ligation to a pGEM-4 vector (Promega, vector digested with the same enzymes) originating pGEM-4-*Hs*-PEX5(C11K)10-324.

The cDNA encoding PEX5ΔN137, which is a protein comprising amino acids 138 to 639 of *Homo sapiens* PEX5 with an upstream initiator methionine, was amplified from pGEM-4-PEX5L(C11K) in order to be inserted in pET23a (Novagen). (see primers in Table 4). The gel-purified cDNA was cloned into the pET-23a NdeI and SalI (Takara) restriction sites, originating pET23a-*Hs*-PEX5ΔN137. Protein i*n vitro* synthesis using this vector is possible due to the T7 promoter it contains. To produce a recombinant version of this protein with a hexahistidine N-terminal tag (His_6), both pET23a-*Hs*-PEX5ΔN137

and pET-28a (Novagen) were digested with NdeI and SalI (Takara). This was followed by gel-purification and ligation of the two molecules, originating pET28a-His_6-*Hs*-PEX5ΔN137. To introduce the recognition sequence for TEV (Tobacco Etch Virus) protease between the histidine tag and the PEX5 moiety, the pET28a-His_6-*Hs*-PEX5ΔN137 was digested with NdeI, dephosphorylated with calf intestine phosphatase (NEB) and ligated to a linker coding for the recognition sequence of the TEV protease (see primers in Table 4). After transformation (see section 3.7) positive clones with single insertions of the linker were selected by colony PCR and 3% agarose/TBE gel analysis. Clones were sequenced to ensure the correct orientation of the linker. Expression and purification of recombinant PEX5ΔN137 was performed by my colleague Ana Dias.

We used a similar strategy (*i.e.*, an equal procedure to the one for creating pET23a-Hs-PEX5ΔN137 and pET28a-His_6-Hs-PEX5ΔN137) to develop pET23a-Hs-PEX5(C11K)1-125 and pET28a-His_6-Hs-PEX5(C11K)1-125 (see primers in Table 4). These latter plasmids encode PEX5(C11K)1-125, which is a protein comprising amino acids residues 1 to 125 of human PEX5 harbouring the mutation C11K. However, production of this protein in *Escherichia coli* (*E. coli*) turn out to be not possible (see below). To solve this problem, a strategy of sequence-and-ligation-independent-cloning (SLIC) was used to generate the same PEX5 protein fused to a SUMO3 N-terminal solubility tag. PCR was performed with primers LP1_F_vector and LP2_R_vector using as template pCoofy6 [119], a plasmid possessing a T7 promoter, a Kozak sequence and encoding a hexahistidine tag and SUMO3 (see primers in Table 4). The insert PEX5(C11K)1-125 was amplified from pGEM-4-PEX5L(C11K) using primers LP1_F_PEX5 and LP2_R_PEX5 (see primers in Table 4). Then, 23 ng of DNA insert and 100 ng of vector were mixed and incubated with 0.19 mg/mL of Recombinase A and RecA buffer (NEB) for 30 minutes at 37 ºC. The reactions were then treated for 1 hour at 37 ºC with DpnI and the DNA was transformed into *E. coli* DH5α competent cells. The correct plasmid pCoofy6-His_6-SUMO3-PEX5(C11K)1-125 was obtained and transformed into competent *E. coli* BL21(DE3) cells for protein expression.

Table 4 - List of forward (F) and reverse (R) primers that were used to produce cDNAs or to construct plasmids. The sequence of the primers presented is orientated 5' to 3'. The reverse complement of the stop codon is underlined.. In bold are the sequences recognized by the respective restriction enzyme mentioned in the primer's name. Regions of homology for SLIC cloning are double underscored or dashed. (Table continues on the next page).

cDNA Plasmid	**Primers forward (F) and reverse (R)**	**Sequence 5' → 3'**
Hs-PEX5(C11K)1-61	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_61R	TCACTCATCTTCAGAAGCTACTCCCAAAGGCTTGG

cDNA Plasmid	Primers forward (F) and reverse (R)	Sequence 5' → 3'
Hs-PEX5(C11K)1-61-M3	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_61-3M_R	TCACATCATCATCTCATCTTCAGAAGCTACTCCCAAAGG
Hs-PEX5(C11K)1-117	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_117R	GGCGAGTCAGTTCTCAGACAAGGCCAAGTCTGC
Hs-PEX5(C11K/A)1-125	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_125R	GCGAGGTCAAGCTGCAAGAAACTCCTGG
Hs-PEX5(C11K/A)1-139	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_139R	GCCGTCAGTCAGTCTCATTATAATCCTGAGTTACATCC
Hs-PEX5(C11K/A)1-158	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_158R	GCAAATCAGCGGGCAGGGGACACAGACAAGG
Hs-PEX5(C11K/A)1-181	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_81R	GCAAATCAGGTGGCTGTTCCCTCAGG
Hs-PEX5(C11K)1-197	pGEM-4APF	GCCCAATACGCAAACCGCCTCTCC
	Pex5_197R	GATAATCACGTGTGCTGCAGATCCTCCTCAGG
pGEM-4-Hs-PEX5(C11K)10-324	PEX5_DN9F_SalI	GGAATAA**GTCGAC**ATGGAAAAGGGGGGTGC
	PEX5_324R_XbaI	CGGGCAGG**TCTAGA**TCAGTACCCCTTATCATAGGTAGC
pET-23a-Hs-PEX5(C11K)1-125	F_1-125_NdeI	GGACACA**CATATG**GCAATGCGGGAGCTGG
	R_1-125_SalI	GGCAAG**GTCGAC**TCAAGCTGCAAGAAACTCCTGG
pET-23a-Hs-PEX5ΔN137	F_138-639_NdeI	GGAACG**CATATG**ACTGACTGGTCCCAAGAATTCATCTC
	R_138-639_SalI	GATAAC**GTCGAC**TCACTGGGGCAGGCCAAACATAG
TEV linker	F_TEV_NdeI	[Phos]**TATG**GAGAACCTTTATTTCCAGGGCCA
	Rv_TEV_NdeI	[Phos]TATGGCCCTGGAAATAAAGGTTCTC**CA**
pCoofy6-His_6-SUMO3-PEX5(C11K)1-125	LP1_F_vector	TCCACCGGTCTGCTGCTGGAACAC
	LP2_R_vector	CGCCATTAACCTGATGTTCTGGGG
	LP1_F_PEX5	GTGTTCCAGCAGCAGACCGGTGGAATGGCAATGCGG
	LP2_R_PEX5	CCCCAGAACATCAGGTTAATGGCGTCAAGCTGCAAGAAACTCC

3.2 Synthesis of ^{35}S-labeled proteins

Plasmids and linear cDNAs encoding the different versions of PEX5 were used for *in vitro* transcription/translation in the presence of EasyTag™ L-[^{35}S]methionine (specific activity >1000 Ci/mmol; PerkinElmer) with the TNT® Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. The proteins obtained were analysed by SDS-PAGE and autoradiography.

3.3 Production and purification of recombinant proteins

GST-Ub, a recombinant fusion protein comprising glutathione-S-transferase and ubiquitin was obtained as previously described [84].

Other recombinant proteins used in this work were already available in our laboratory. These proteins are: 1) recombinant TPRs [101], a protein comprising PEX5L residues 315 to 639; 2) recombinant TPRs(N526K) [116], a protein containing amino acids 315 to 639 of PEX5L with the missense mutation N526K; and 3) recombinant cSENP2 [120,121], the catalytic domain of SENP2.

To produce recombinant His_6-TEV-PEX5(C11K)1-125 various experimental conditions were attempted. These included: induction of *E. coli* BL21(DE3) cultures for 3 hours with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 ºC and induction with 10 µM or 1 mM IPTG for 18 hours at 17 ºC. We also tried to pre-induce the chaperone machinery of *E. coli* by incubating bacterial cultures for 30 minutes on ice followed by the addition of 2% ethanol and induction with IPTG (0.5 mM for 2 hours at 37 ºC). Alternatively, bacterial cultures were treated for 5 minutes at 47 ºC before proceeding with the IPTG induction (0.5 mM for 2 hours at 37 ºC). The latter two protocols were also tested using different bacterial growth stages (OD_{600nm} = 0.6 or 0.9). Protein expression was assessed by SDS-PAGE and Western Blot analysis using an anti-His antibody (GE Healthcare). Since none of these attempts yielded positive results, we tried to produce PEX5(C11K)1-125 from a different expression plasmid, the pCoofy6-His_6-SUMO3-PEX5(C11K)1-125 described above. For this purpose, *E. coli* BL21(DE3) cells carrying this plasmid were induced for 2 hours at 37 ºC with 1 mM IPTG and cooled on ice. Next, cells were pelletized by centrifugation at 3500 rpm at 4 ºC for 20 minutes (Beckman Avanti J-26 XP, rotor JLA 8.1000). The pellet was resuspended in 20 mL of lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1 mM Dithiothreitol (DTT), 1:500 (v/v) Phenylmethanesulfonyl fluoride (PMSF) 50 mg/mL, 1:500 (v/v) mammalian protease inhibitor cocktail (Sigma), 200 µg/mL lysozyme] and frozen overnight at -80 ºC. Cells were thawed, sonicated on ice, and the lysate was centrifuged at 13000 rpm for 20 minutes at 4 ºC (Thermo Scientific Sorval Legend Micro 17 centrifuge). The supernatant was incubated with 400 µL (bed volume) of Ni^{2+}-NTA Sepharose 6 Fast Flow beads (GE Healthcare) for 3 hours at 4 ºC with mixing. The non-bound fraction was removed, and the beads were washed three times with wash buffer [50 mM phosphate buffer pH 8.0, 150 mM NaCl]. The protein was eluted with elution buffer [50 mM phosphate buffer pH 8.0, 150 mM NaCl, 400 mM imidazole] and residual beads were removed by centrifuging the protein solution at 13000 rpm for 10 minutes at 4 ºC (Thermo Scientific Sorval Legend Micro 17 centrifuge). Concentration of the recombinant protein and buffer exchange to

the storage buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT] was achieved using a Vivaspin 2 MWCO 10 000 protein concentrator (GE Healthcare), as described recently [76].

Removal of the His_6-SUMO3 moiety from the recombinant protein His_6-SUMO3-PEX5(C11K)1-125 was done by digesting 1.2 milligrams of the purified recombinant protein with 1.2 µg of cSENP2 at 25 ºC for 1 hour. The solution was then incubated with 133 µL (bed volume) of Ni^{2+}-NTA Sepharose 6 Fast Flow beads (GE Healthcare) for 3 hours at 4 ºC with mixing. After sedimentation of the beads, the non-bound fraction, containing the protein of interest was saved. During this procedure, aliquots were removed at different steps and analysed by SDS-PAGE to control the purification process.

3.4 Preparation of rat liver post-nuclear supernatant

A post-nuclear supernatant (PNS) from rat liver was prepared as described before [83]. Briefly, livers from overnight fasted Wistar male rats (1-2 months old) were removed and homogenized in ice-cold SEM buffer [0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, and 1 mM EDTA-NaOH, pH 8.0 supplemented with 2 µg/ml N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64)]. The homogenate was centrifuged twice at 2200 rpm for 10 min at 4 °C (SS-34 rotor in a RC5B Sorvall® centrifuge) to yield a post-nuclear supernatant fraction which was aliquoted, frozen on liquid nitrogen and stored at -80 ºC. The PNS protein concentration was determined by the Bradford method.

3.5 *In vitro* import/export experiments

A typical reaction is performed in a final volume of 100 µL and contains 600 µg of PNS protein, and 1 to 3 µL of rabbit reticulocyte lysate (RRL) containing the relevant ^{35}S-labelled protein in import buffer [0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH pH 7.2, 3 mM $MgCl_2$, 20 µM methionine, 2 µg/mL E-64, and 2 mM GSH, pH 7.2]. Where indicated, the reactions were supplemented with 3 mM ATP or 3 mM AMP-PNP, 15 µM of bovine ubiquitin (Ub) or GST-Ub, 0.8 µM of HA-Ubiquitin Aldehyde (HA-Ubal) or 2 µM of HA-Ubiquitin-vinyl methyl ester (HA-Ub-VME), 1 µM of rTPRs or 1 µM rTPRs(N526K). Reactions were incubated at 37 ºC for 20 minutes, unless otherwise specified. In the experiments containing AMP-PNP, the PNS was first primed for import by incubating it with 0.3 mM ATP at 37ºC for 5 minutes. This step allows for endogenous DTM-embedded PEX5 to be exported back into the soluble fraction, therefore increasing the number of available free DTMs and consequently enhancing the import efficiency of the ^{35}S-PEX5 proteins [78,80].

Reactions were stopped by putting them on ice. When the aim was to monitor import/export of ^{35}S-PEX5 proteins, the reactions were diluted with SEMK buffer (SEM buffer containing 80 mM KCl) to 1 mL and centrifuged at 13000 rpm for 20 minutes at 4 ºC (Thermo Scientific Sorval Legend Micro 17 centrifuge), yielding an organelle pellet (P) and a supernatant (S) which corresponds to the cytosolic fraction. Proteins in the supernatant fraction from import/export assays were precipitated with TCA by adding 1/9th of the sample volume of 100% (w/v) TCA and incubating on ice for 30 minutes. Precipitated proteins were then recovered by centrifugation (13000 rpm for 20 minutes at 4 ºC - Thermo Scientific Sorval Legend Micro 17 centrifuge), washed with 1 mL of 100% acetone, centrifuged (13000 rpm for 20 minutes at 4 ºC - Thermo Scientific Sorval Legend Micro 17 centrifuge) and dried at 37 ºC.

When the aim was to assess the membrane topology of ^{35}S-labelled PEX5 proteins, reactions were treated on ice with 400 µg/mL of Proteinase K (PK) for 30 minutes (unless otherwise indicated). During this incubation, susceptible proteins will be degraded only if they are accessible to the protease; if, on the contrary, a given protein is inside an intact organelle, it will escape degradation because the protease cannot cross the lipid membrane. At the end of the incubation, PK was inactivated with 400 µg/mL of PMSF, reactions were diluted to 1 mL with SEMK buffer and centrifuged at 13000 rpm for 20 minutes at 4 ºC (Thermo Scientific Sorval Legend Micro 17 centrifuge). Organelle pellets were subjected to precipitation with a 10% (w/v) TCA solution and processed as described above.

3.6 SDS-PAGE and autoradiography

Protein samples were heated in Laemmli sample buffer [50 mM Tris-HCl pH 8.8, 6% SDS, 0.017% bromophenol blue, 10% glycerol, 2 mM EDTA-NaOH pH 8.8, supplemented with 0.1 M DTT] for 10 minutes at 65 ºC followed by 5 minutes at 90 ºC with shaking. The samples were loaded onto SDS polyacrylamide gels using a discontinuous electrophoretic system [122]. Gels were blotted onto nitrocellulose membranes (Amersham™ Protran™ 0.45 µM GE Healthcare) using a semi-dry system (The W.E.P. Company) according to the manufacturer instructions. The membranes were stained with Ponceau S stain [0.026 M Ponceau S, 1.84 M TCA in deionized water], dried and exposed to a x-Ray film (Carestream BioMAX MR film, Cat 873 6936).

3.7 Miscellaneous

All the recombinant plasmids constructed in this work by standard ligation procedures were transformed into NovaBlue GigaSingles™ Competent Cells (Novagen).

Positive clones were identified by colony PCR screening. Plasmid DNA from these clones was prepared using NZYMiniprep kit (MB01001) and sequenced (GATC Biotech).

Western blots were probed with a mouse monoclonal anti-His antibody (GE Healthcare 27-4710-01) as the primary antibody, which was detected using alkaline phosphatase-conjugated anti-mouse immunoglobulins G (A2429, Sigma).

4. RESULTS

4.1 Experimental approaches

One of the most powerful experimental tools to dissect the mechanism of the PIM is a cell-free *in vitro* import/export system developed in our laboratory [76,78,83]. In this *in vitro* system, a rat/mouse liver PNS (which contains peroxisomes and cytosolic components) is incubated with a radiolabelled reporter protein in an appropriate buffer. A variety of reporter proteins can be used in these assays, from PEX5 or mutant versions of it, to PEX7, PTS1 and PTS2 proteins [76]. Also, because this is a completely open experimental system numerous reagents (*e.g.*, ATP, AMP-PNP) or recombinant proteins (*e.g.*, GST-Ub) can be added to these assays [74,76,78,80,118]. Therefore, one can obtain rather different but complementary perspectives on how the PIM works [70,74,76,78,83,84]. In this work, we used PEX5-centered *in vitro* import assays to dissect the structural/functional relationships in PEX5. When using PEX5 as a reporter protein, two experimental readouts are possible. In one, we can assess the fraction of PEX5 that was inserted into the DTM by performing protease-protection experiments after an *in vitro* import assay. Two populations of PEX5 can be detected using this approach [75,78]. The so-called stage 2 represents a PEX5 molecule having most of its polypeptide chain exposed into the peroxisomal lumen, and thus, protease-protected. In fact, only a few residues of stage 2 PEX5 can be removed by proteinase K digestion when using intact organelles [77,83]. The second DTM-embedded PEX5 population – the so-called stage 3 PEX5 – represents monoubiquitinated PEX5 and is completely resistant to proteinase K [83,84]. Note that stage 2 and stage 3 PEX5 probably have the same membrane topology; the proteinase K resistance of stage 3 PEX5 is best explained by a shielding effect exerted by the ubiquitin moiety attached to PEX5 or by the REM itself [83,84].

The second readout is based on the fact that PEX5 is monoubiquitinated only after insertion into the DTM and can be exported back into the cytosol provided that the system contains ATP; in the presence of non-hydrolysable ATP analogues, such as AMP-PNP or ATPγS, monoubiquitination of PEX5 at the DTM is still possible but its export by the REM is completely blocked [78,84]. Thus, a simple centrifugation of import reactions to separate organelles from the cytosolic phase followed by SDS-PAGE/autoradiography of the two fractions (often referred to simply as P (pellet) and S (supernatant)) provides valuable information on the functionality of a given PEX5 molecule [76].

Monoubiquitination of PEX5 occurs at cysteine 11, which yields a thioester ubiquitin-PEX5 conjugate [84]. Thioesters are relatively labile and tend to hydrolyse at the alkaline pH of the SDS-PAGE. Also, they are completely destroyed by reducing

agents (*e.g.*, DTT and β-mercaptoethanol), complicating the experimental procedure to detect them [84]. In 2009 Grou *et al.*, described that a PEX5 mutant protein with the cysteine 11 substituted by a lysine (C11K) is fully functional in import, ubiquitination and export. PEX5(C11K) was also shown to complement the import of PTS1-cargo proteins in PEX5 deficient mouse embryonic fibroblasts [89]. Since ubiquitin is linked to PEX5(C11K) by an isopeptide bond (which is not disrupted by reducing agents/alkaline pH), most of the proteins used in this work derived from PEX5(C11K) [89].

Several truncated PEX5 molecules were produced in this work. In addition to various PEX5(C11K) mutant forms, the corresponding C11A versions were also created. PEX5 proteins possessing an alanine at position 11 cannot undergo monoubiquitination and therefore are not exported back to the cytosol [89]. Thus, they accumulate at peroxisomal membrane DTMs even when *in vitro* assays are performed in the presence of ATP. This makes them much easier to detect after protease treatment. The structural details of all the different PEX5 molecules used in this work are presented in Fig. 4 and Table 5.

Table 5 - Properties of the PEX5 proteins used in this work.
The molecular weight in kilodaltons (kDa) of each protein was calculated in Protparam [100], the number of amino acid residues and pentapeptide motifs that each protein contains are also provided.

PEX5 protein	Predicted molecular weight (kDa)	Number of Residues	Pentapeptide motifs
PEX5(C11K)1-61	6.397	61	----------
PEX5(C11K)1-61-M3	6.790	64	----------
PEX5(C11K)1-117	12.62	117	0
PEX5(C11K/A)1-125	13.54	125	0,1
PEX5(C11K/A)1-139	15.06	139	0,1
PEX5(C11K/A)1-158	17.19	158	0,1,2
PEX5(C11K/A)1-181	19.87	181	0,1,2,3
PEX5(C11K/A)1-197	21.98	197	0,1,2,3,4
PEX5(C11K/A)1-324	36.17	324	0,1,2,3,4,5,6,7
PEX5L(C11A/K)	70.90	639	0,1,2,3,4,5,6,7
PEX5ΔN137	56.93	503	2,3,4,5,6,7
PEX5ΔN147	54.87	492	3,4,5,6,7
PEX5(C11K)10-324	35.37	317	0,1,2,3,4,5,6,7

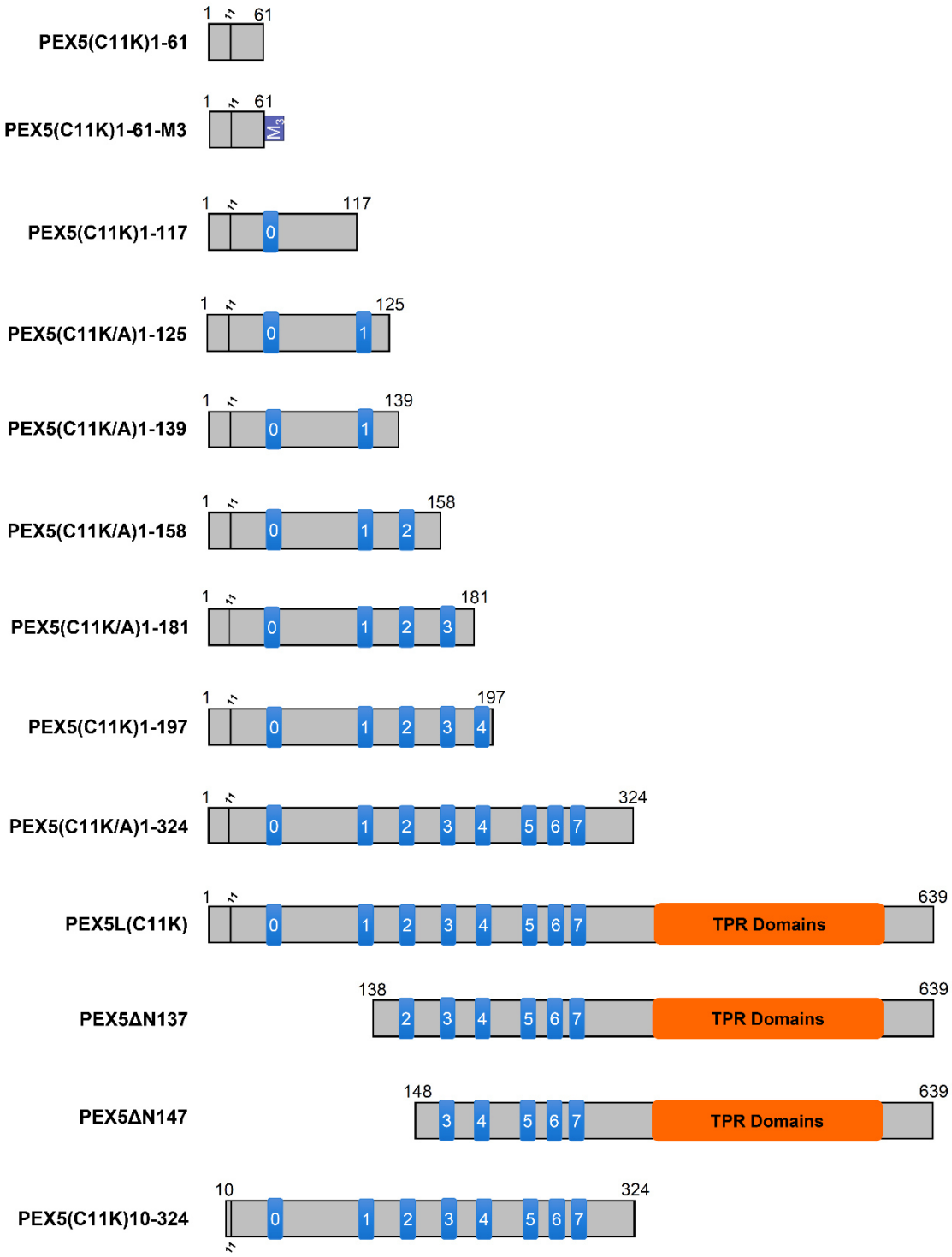

Fig. 4 - PEX5 truncations for *in vitro* import/export assays.
Deletion mutants of PEX5 were constructed by PCR using as template either PEX5L(C11K) or PEX5L(C11A) as indicated, the position 11 is specified in the representations (for more detail see experimental procedures section 3.1). cDNAs were purified and either directly used for *in vitro* transcription/translation (IVT) in a rabbit reticulocyte lysate (RRL) or cloned into *E. coli* expression vectors. The PEX5 residues contained in each deletion mutant are indicated. The truncations vary in the number of pentapeptide motifs (ranging from 0 to 7, blue) and in the presence of the TPRs motifs (orange, "TPRs"). The presence of 3 tandem methionines (M3) is represented in purple.

4.2 PEX5ΔN137 is imported in a cargo-dependent manner

As stated in the introduction section, insertion of PEX5 into the DTM is a cargo-dependent event [75]. This property probably reflects the existence of an autoregulatory mechanism in PEX5 itself. Our hypothesis is that the C-terminal TPRs domain of PEX5 is a *cis*-acting repressor of its N-terminal half, the domain where the DTM interacting motifs of PEX5 reside. We knew from previous work that a PEX5 protein lacking the first 110 amino acids is still a robust substrate for the peroxisomal DTM. Importantly, insertion of this mutant PEX5 is cargo-protein dependent, implying that the first 110 amino acids residues of PEX5 are not part of this autoregulatory mechanism [73]. To better define the PEX5 region(s) involved in this mechanism, we used two truncated PEX5 molecules lacking larger N-terminal regions in *in vitro* import assays. The aim was two-fold: 1) to define the smallest PEX5 protein that is still import competent in a cargo-dependent manner; and 2) try to identify a PEX5 protein still capable of interacting with PTS1-proteins and entering the DTM, but displaying the capacity to enter the DTM in a cargo-independent manner.

Three ^{35}S-labelled PEX5 proteins were used in these experiments: PEX5L(C11K) as a positive control, PEX5ΔN137 and PEX5ΔN147 (see Fig. 4). *In vitro* import assays were done in the presence of rTPRs or rTPRs(N526K). rTPRs binds PTS1-cargo proteins present in the PNS but cannot be targeted to the peroxisomal membrane. Under these conditions, the ^{35}S-labelled PEX5 reporter proteins cannot bind PTS1-proteins, and therefore do not become inserted into the DTM [75]. rTPRs(N526K) is virtually identical to rTPRs with the exception that it possesses a lysine instead of an asparagine at position 526. This missense mutation completely abolishes its PTS1-binding activity, and thus this protein is used as a negative control for rTPRs [59,116]. At the end of the import reaction, organelle suspensions were treated with a vast amount of proteinase K (PK). PK degrades all non-imported and non-specifically adsorbed radiolabelled proteins, whereas DTM inserted ones remain resistant [75,76,78,83].

As shown in Fig. 5, ^{35}S-PEX5L(C11K) acquired a protease-protected status only in the assay lacking rTPRs, implying that this protein enters into the DTM in a cargo-dependent manner, as expected (panel A, compare lanes “rTPRs” with “rTPRs(N526K)”). An identical result was obtained for ^{35}S-PEX5ΔN137, clearly showing that this protein is also inserted into the DTM in a cargo-dependent manner (panel B). Note that more PK-resistant PEX5ΔN137 than PEX5(C11K) is detected in these experiments. This is due to the fact that N-terminally truncated PEX5 proteins lacking a ubiquitinatable residue at position 11 are export-incompetent [73]. For ^{35}S-PEX5ΔN147 a different result was obtained – no, or almost no, protein entered the DTM. We did detect

some signal after a very long exposure of the nitrocellulose membrane to an X-ray film. However, no evidence for cargo-dependence was obtained. (Note, that although the radioactive signal of ^{35}S-PEX5ΔN147 is slightly stronger in the reaction performed in the presence of rTPRs(N526K) than in the presence of rTPRs, this difference is due to a larger protein load in former – see the Ponceau S stained membrane in panel C).

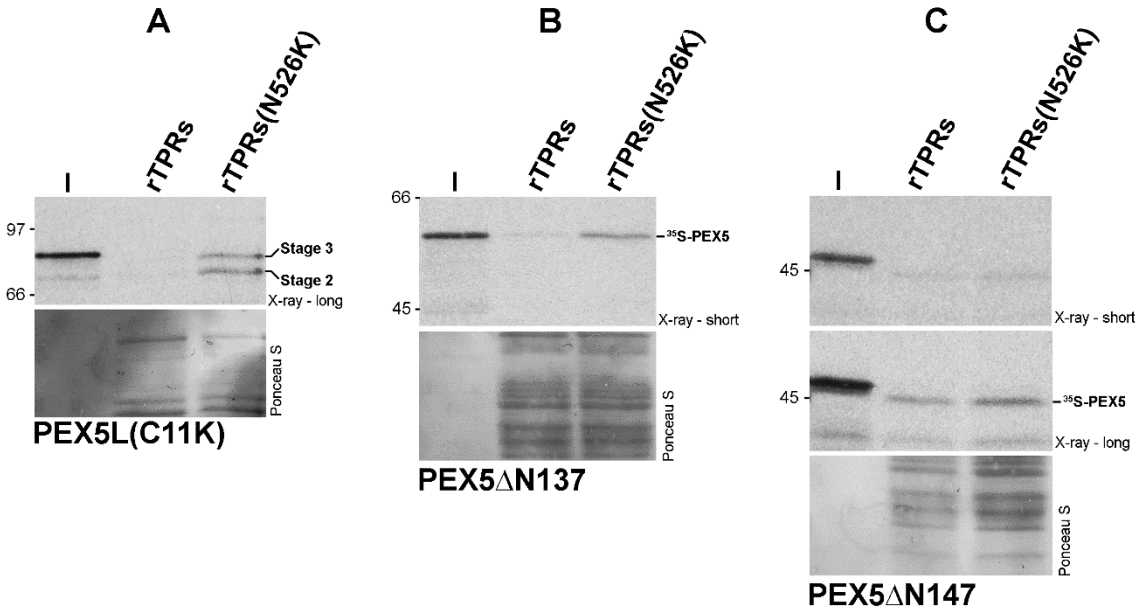

Fig. 5 - Entry of ^{35}S-PEX5ΔN137 in the peroxisomal membrane is a cargo-dependent process.
Import reactions containing 600 µg of PNS protein were incubated with PEX5L(C11K) **(A)**, ^{35}S-PEX5ΔN137 **(B)** or ^{35}S-PEX5ΔN147 **(C)**, ATP and **rTPRs** or **rTPRs(N526K)**. After import, proteinase K-treated organelles were isolated and analysed by SDS-PAGE/autoradiography (X-ray – short is an exposure of 16 hours (B) or 4 days (A and C), X-ray – long is an exposure of 16 days). **Lanes I**, represent 10% of the ^{35}S-PEX5 used in each reaction. **^{35}S-PEX5**, indicates the radiolabeled PEX5 protease-protected species after the *in vitro* import assay. Numbers on the left indicate the molecular mass of the protein standards in kDa.
Note that for imported PEX5L(C11K), protease treatment results in two protease-protected species, namely **stage 2** and **stage 3** PEX5, which are indicated on the right (**A**). Recall from the introduction that stage 2 PEX5 represents DTM-embedded PEX5 molecules that expose to the cytosol a N-terminal fragment. This region of PEX5 is clipped by PK, yielding a protein shorter by *ca.* 2 kDa. Stage 3 PEX5 represents the pool of monoubiquitinated DTM-embedded PEX5 where the cytosolic N-terminal region of PEX5 is protected from the PK action and thus, no molecular wieght shift is observed.

Taken together, these results suggest that the first 137 amino acids of PEX5 are important neither for the docking/insertion of PEX5 into the DTM nor for its cargo-dependent regulation. In addition, these results may suggest that amino acid residues 138 to 147 of PEX5 are crucial for DTM insertion of PEX5 proteins containing their PTS1-binding domain (TPRs). Unfortunately, the latter result also means that we could not identify a PEX5 protein shorter that PEX5ΔN137 that is still import-competent but now in a cargo-independent manner.

4.3 The first 117 amino acid residues of PEX5 are sufficient for its import and ubiquitination

The interaction of PEX5 with DTM components involves the N-terminal half of PEX5 which contains the 8 pentapeptide repeats referred to previously (see introduction section 1.4) [34,112–114]. Indeed, this domain of PEX5 is crucial for its docking at and insertion into the DTM (see also introduction section 1.4). For example, PEX5 molecules lacking the TPRs domain but containing residues 1-197 or 1-324 of PEX5 are fully capable of being inserted into the DTMs as assessed by protease-protection experiments [75]. Interestingly, PEX5(1-197) contains just pentapeptide motifs 0 to 4. More recently, transfection experiments using CHO/HeLa cells led some authors to propose that a truncated PEX5 comprising just its first 158 amino acid residues is also functional in the interaction with the peroxisomal import machinery, although details on this interaction were not provided [123]. Aiming at defining the shortest PEX5 N-terminal fragment having the necessary and sufficient information to be 1) imported, 2) ubiquitinated, and 3) exported, we produced and characterized *in vitro* several C-terminally truncated PEX5(C11K) proteins shorter than the ones described above.

In addition to PEX5(C11K)1-324 and PEX5(C11K)1-197, used here as positive controls, seven different ^{35}S-PEX5 proteins were studied. These differ mainly by the number of pentapeptide motifs they contain. Thus, PEX5(C11K)1-181 contains pentapeptides 0-3, PEX5(C11K)1-158 contains pentapeptides 0-2, PEX5(C11K)1-139 and PEX5(C11K)1-125 contain pentapeptides 0-1, PEX5(C11K)1-117 contains only pentapeptide 0, and finally PEX5(C11K)1-61 contains no pentapeptide motifs (see Fig. 4 for a graphical representation of all these molecules).

In order to determine the import/ubiquitination ability of the above referred proteins we performed *in vitro* assays in the presence of AMP-PNP, an ATP analogue which can be used by the ubiquitin-conjugating cascade (UCC) but not by the receptor export module [97,124]. Under these conditions, import and ubiquitination of PEX5 proteins is rather efficient but their export is not possible, thus simplifying analysis of the results (only organelle pellets need to be analysed) [89]. Specific ubiquitination of PEX5 proteins was assessed in two different ways: 1) by a *ca.* 8 kDa increase in the apparent molecular mass of the PEX5 proteins upon SDS-PAGE when bovine ubiquitin was used in these assays; or 2) by a *ca.* 32 kDa increase when GST-Ub was used instead. (Note that GST-Ub is also used efficiently by the UCC acting on PEX5. However, it results in PEX5 species which are no longer substrates for the REM [84]).

As shown in Fig. 6, all C-terminally truncated PEX5 proteins but one were ubiquitinated in these assays; the exception was PEX5(C11K)1-61. Thus, a single pentapeptide motif, namely pentapeptide 0, seems to be sufficient to ensure that PEX5(C11K)1-117 enters the DTM and is monoubiquitinated. We still considered the possibility that PEX5(C11K)1-61 monoubiquitination might not be detectable in these assays due to the small number of methionines present in this PEX5 domain (the

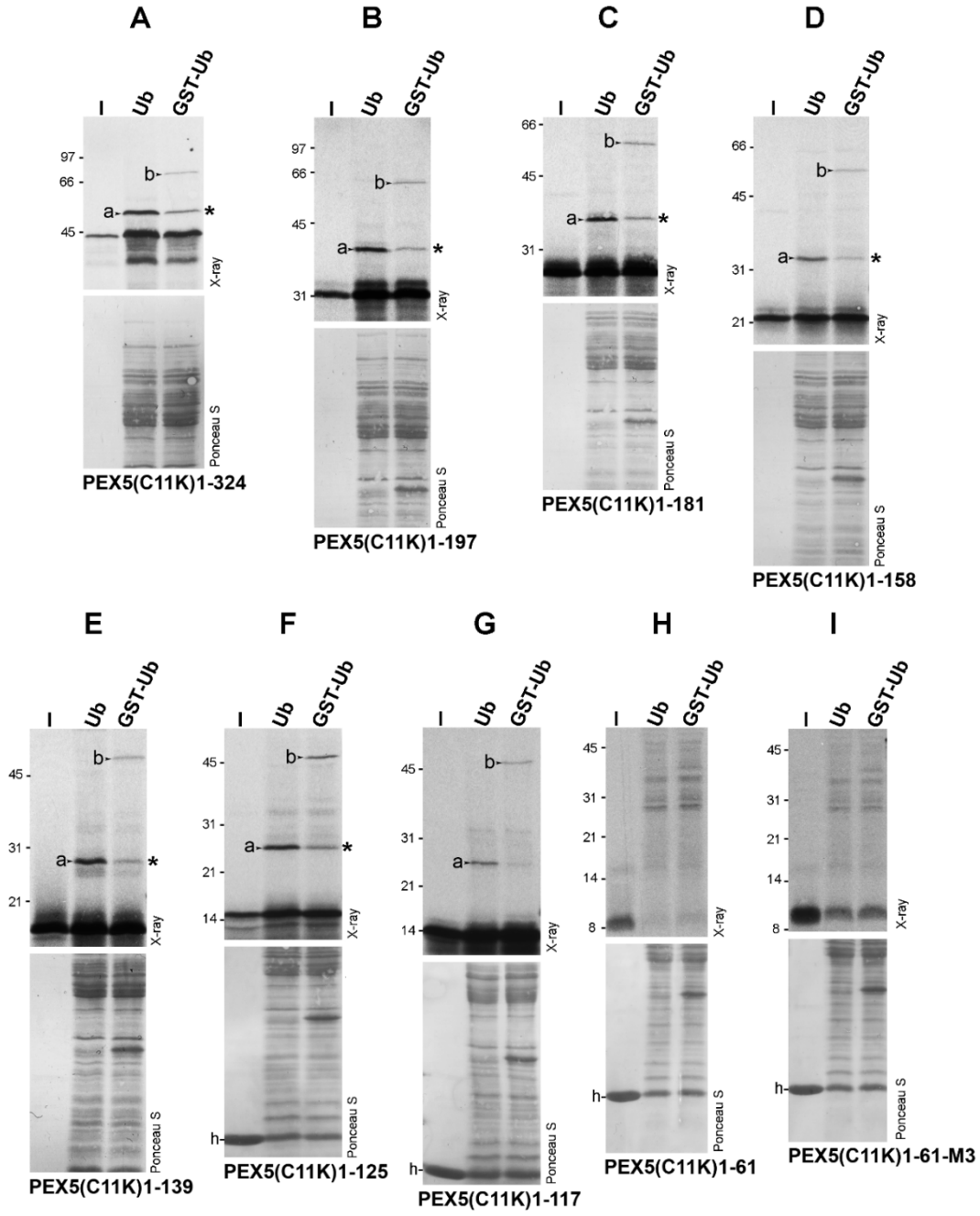

Fig. 6 - *In vitro* ubiquitination assay of C-terminal PEX5 truncations.
Import reactions containing 600 µg of primed PNS protein were incubated with one of the following proteins ^{35}S-PEX5(C11K)1-324 (**A**), ^{35}S-PEX5(C11K)1-197 (**B**), ^{35}S-PEX5(C11K)1-181 (**C**), ^{35}S-PEX5(C11K)1-158 (**D**), ^{35}S-PEX5(C11K)1-139 (**E**), ^{35}S-PEX5(C11K)1-125 (**F**), ^{35}S-PEX5(C11K)1-117 (**G**), ^{35}S-PEX5(C11K)1-61 (**H**), ^{35}S-PEX5(C11K)1-61-M3 (**I**) in the presence of AMP-PNP and either ubiquitin (lanes **Ub**) or GST-Ubiquitin (lanes **GST-Ub**). Organelle pellets (P) were isolated and prepared for SDS-PAGE/autoradiography **Lanes I**, represent 10% of the ^{35}S-protein used in each reaction. **a**, Ub-PEX5 species. **b**, GST-Ub-PEX5 species. *****, Ub-PEX5 modified with endogenous ubiquitin. **h**, haemoglobin from the RRL. Numbers on the left indicate the molecular mass of the protein standards in kDa.

radiolabelled amino acid used in our IVT is ^{35}S-methionine and PEX5(C11K)1-61 contains only 3 methionine residues). To exclude this, we produced a PEX5(C11K)1-61 protein possessing 3 extra methionines at its C-terminus. As shown in Fig. 6 panel I, no specific ubiquitination of this PEX5 protein was observed.

In short, we have narrowed down the minimum sequence of PEX5 necessary for its peroxisomal association (docking), insertion and monoubiquitination at the DTM to the amino acid residues 1 to 117.

4.4 Ub-PEX5(C11K)1-117 is a substrate for the receptor export module

Recycling of DTM-embedded PEX5 back into the cytosol was shown to be dependent on: 1) the monoubiquitination of the receptor itself, and 2) hydrolysis of ATP [78,84,89]. These findings suggested that the AAA ATPases PEX1 and PEX6, the only two peroxins that bind and hydrolyse ATP, are involved in the extraction of Ub-PEX5 from peroxisomal membranes [78,125]. However, on the actual mechanism used by the AAA ATPases to extract Ub-PEX5 not much is known. Thus, we reasoned that it would be interesting to know whether the small N-terminal PEX5 proteins characterized above and shown to be ubiquitinated are still substrates for the REM. These experiments might reveal the existence of PEX5 regions with no role in docking, insertion into the DTM and ubiquitination, but of crucial importance for the interaction between PEX5 and the REM. Anyway, even if it turns out that these PEX5 regions do not exist, assessment of the export-competence of those small N-terminal PEX5 proteins will lead to a better definition of the minimal domain of PEX5 that is necessary for the export step. (It should be noted that the shortest PEX5 protein, presently known, that is still functional in the import and export steps is PEX5(1-324) [73]).

For this purpose, we subjected three of the small ubiquitinatable PEX5 proteins identified above, *i.e.,* PEX5(C11K)1-117, PEX5(C11K)1-125 and PEX5(C11K)1-158, to *in vitro* reactions using experimental conditions in which export can or not occur (assays performed in the presence of ATP or AMP-PNP, respectively). HA-Ubal (or HA-Ub-VME) was included in these assays to protect exported (soluble) Ub-PEX5 proteins from the action of deubiquitinases present in the soluble phase of PNS [97]. These modified ubiquitin molecules are potent inhibitors of deubiquitinating enzymes [126].

As shown in Fig. 7 (panel A), ^{35}S-PEX5(C11K)1-324 was monoubiquitinated both in the presence of ATP and AMP-PNP, but this PEX5 protein was only extracted from the DTM when ATP was available, as expected (compare lanes “P” and “S” under AMP-PNP and ATP) [73,78]. Significantly, all the other tested PEX5 truncations, *i.e.,* ^{35}S-PEX5(C11K)1-158, ^{35}S-PEX5(C11K)1-125 and ^{35}S-PEX5(C11K)1-117 (Fig. 7, panel B, C and D, respectively), were exported from the organelles in the *in vitro* import assays provided that export-permissive conditions were employed (compare organelle pellet “P” with supernatant “S” of ATP-containing reactions). Indeed, in the presence of AMP-PNP, all Ub-PEX5 species remained associated with the organelles, clearly showing that ATP hydrolysis is necessary to extract these PEX5 proteins from the DTM (compare lanes “P” and “S” under AMP-PNP) [78,84].

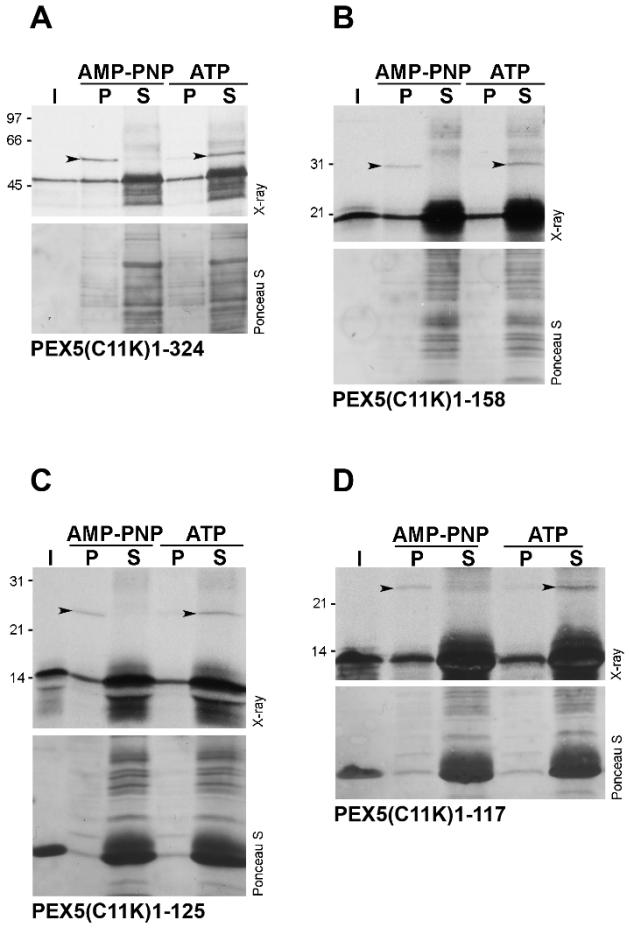

Fig. 7 - *In vitro* export of ^{35}S-PEX5(C11K) C-terminal deletions.
Import reactions containing 600 µg of primed PNS protein were incubated with ^{35}S-PEX5(C11K)1-324 as a control (**A**), ^{35}S-PEX5(C11K)1-158 (**B**), ^{35}S-PEX5(C11K)1-125 (**C**) or ^{35}S-PEX5(C11K)1-117 (**D**), ubiquitin and HA-Ubal (A, B and C) or HA-Ub-VME (D). Each construct was imported in presence of **AMP-PNP** or **ATP**. After import, reactions were fractionated into an organelle pellet (**P**) and supernatant (**S**), and the equivalent to 100 µg of PNS protein was analysed by SDS-PAGE/autoradiography. Equivalents of 150 µg of PNS protein from the PEX5(C11K)1-117 reactions were loaded. **Lanes I**, represent 10% of the ^{35}S-protein used in each reaction. ➤, indicates monoubiquitinated PEX5. Numbers on the left indicate the molecular mass of the protein standards in kDa.

These results, together with the observations made in section 4.3, indicate that amino acids 1-117 of PEX5 are sufficient for its peroxisome-cytosol trafficking. Thus, if there is indeed an interaction between PEX5 and the REM crucial for the PEX5 export,

then this interaction involves some domain within the first 117 amino acid residues of PEX5.

4.5 The first 9 amino acid residues of PEX5 are not important for its function

The alignment of the amino acid sequences of PEX5 from several organisms reveals a highly conserved C-terminal half (the TPRs containing domain which binds the PTS1 signal) and a highly divergent N-terminal domain [54]. In fact, besides the evolutionarily conserved cysteine and some pentapeptide motifs, the positioning of which varies substantially among organisms, not much else was evolutionary preserved. Nevertheless, sequence analysis of PEX5 proteins from Metazoa and also some Plants shows that the residues that precede the conserved cysteine residue at the N-terminus tend to be reasonably conserved (see Fig. 8). This property might suggest that these residues are functionally important. We know from previous studies that these N-terminally located residues of PEX5 are not important for cargo-binding, docking at and insertion into the DTM, because a PEX5 protein lacking the first 110 amino acids is completely functional in all these steps [73]. However, they might be important for the interaction of PEX5 with the RING-ligase that monoubiquitinates it or, alternatively for the export step by providing a binding interface for the REM. We decided to address these possibilities.

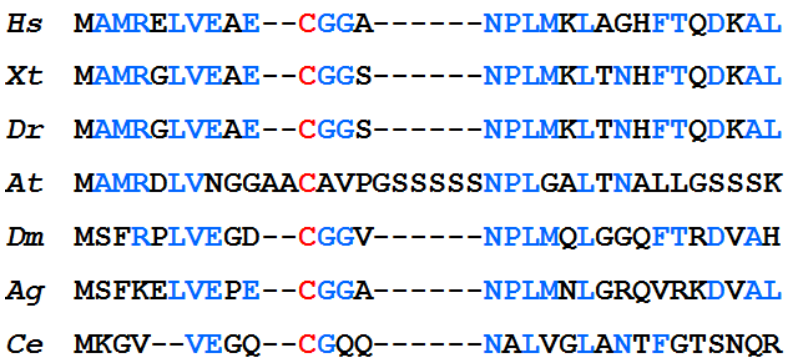

Fig. 8 - Alignment of the PEX5 N-terminal domain containing the conserved cysteine from several organisms. The first amino acid residues of PEX5 from *Homo sapiens* (*Hs*) are aligned with the corresponding regions of PEX5 from *Xenopus tropicalis* (*Xt*), *Danio rerio* (*Dr*), *Arabidopsis thaliana* (*At*), *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*) and *Caenorhabditis elegans* (*Ce*). Residues conserved in four or more sequences are highlighted in blue. The invariable cysteine residue is in red. The PEX5 sequences were obtained from [129].

Firstly, we created a PEX5 protein lacking the first 9 amino acid residues, PEX5(C11K)10-324. The ability of this radiolabelled PEX5 protein to be monoubiquitinated and recycled back into the cytosol was then assessed in *in vitro* import/export experiments identical to the ones presented above (sections 4.3 and 4.4).

In vitro assays were programmed with ^{35}S-PEX5(C11K)10-324 or ^{35}S-PEX5(C11K)1-324, in the presence of AMP-PNP, and either Ub or GST-Ub. The results are shown in Fig. 9 (upper panels). Similarly to ^{35}S-PEX5(C11K)1-324 (panel A), ^{35}S-PEX5(C11K)10-324 (panel B) was monoubiquitinated in these assays, as evidenced by the shift in the molecular weight of the ubiquitinated PEX5 species when GST-Ub was used (compare lanes "Ub" with lanes "GST-Ub"). Overall, these results suggest that the first 9 amino acids of PEX5(C11K) are not important for its import and, despite being adjacent to the modified residue (which in this case is a lysine), are also not required for PEX5 monoubiquitination in mammalian peroxisomes.

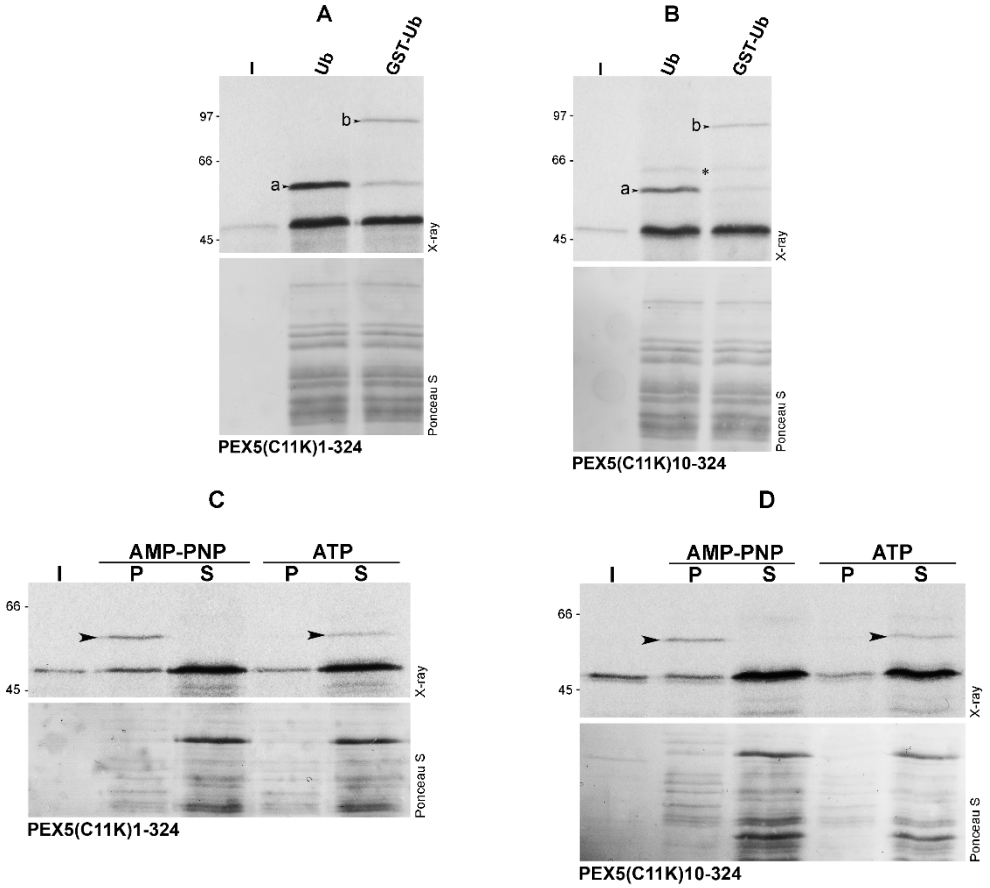

Fig. 9 - Radiolabelled PEX5(C11K)10-324 is an import and export competent protein.
A, B - Import reactions containing 600 µg of primed PNS protein were incubated with ^{35}S-PEX5(C11K)1-324 (**A**) or ^{35}S-PEX5(C11K)10-324 (**B**) in the presence of AMP-PNP and either ubiquitin (lanes **Ub**) or GST-Ubiquitin (lanes **GST-Ub**). Organelle pellets (P) were isolated and prepared for SDS-PAGE/autoradiography. **Lanes I**, represent 10% of the ^{35}S-protein used in each reaction. **a**, represents Ub-PEX5 species; **b**, represents GST-Ub-PEX5 species. *, this band probably derives from an oxidative adduct of the ^{35}S-proteins and the haemoglobin from the RRL.
C, D - Import reactions containing 600 µg of primed PNS protein were incubated with ^{35}S-PEX5(C11K)1-324 (**C**) or ^{35}S-PEX5(C11K)10-324 (**D**), ubiquitin and HA-Ubal. Each construct was imported in presence of **AMP-PNP** or **ATP**. After import, reactions were fractionated into an organelle pellet (**P**) and supernatant (**S**), and the equivalent to 100 µg of PNS protein was analysed by SDS-PAGE/autoradiography. **Lanes I**, represent 10% of the ^{35}S-protein used in each reaction. ➤, ubiquitinated PEX5 species.
Numbers on the left indicate the molecular mass of the protein standards in kDa.

We then assessed whether the first N-terminal residues of PEX5 are involved in the recognition and/or extraction of Ub-PEX5 by the REM. For this purpose, the subcellular localization of ^{35}S-Ub-PEX5(6C11K)10-324 was examined following *in vitro* import assays done in the presence of either AMP-PNP or ATP. Fig. 9, panel D, shows

that in export-permissive conditions (*i.e.*, ATP) ^{35}S-Ub-PEX5(C11K)10-324 is recovered in the cytosolic fraction, behaving exactly as the control protein ^{35}S-PEX5(C11K)1-324 (panel C) [73,90].

Altogether, these experiments indicate that the first 9 amino acids of human PEX5 are not necessary for the transient passage of PEX5 through the peroxisomal docking/translocation module. Together with the results described in previous sections, we can conclude that the minimal PEX5 domain necessary and sufficient for DTM insertion, monoubiquitination and extraction back into the cytosol comprises amino acids 10 to 117 of PEX5.

4.6 Membrane topology assessment of C-terminally truncated PEX5 molecules

To better define the interaction between the DTM and the small N-terminal PEX5 proteins characterized above, we decided to assess their membrane topologies using protease-protection assays. Recall that when full-length PEX5 is analysed using this methodology, two DTM-embedded PEX5 pools are discerned: 1) non-ubiquitinated PEX5 (the so-called stage 2 PEX5) from which only a 2 kDa N-terminal segment can be removed by the protease, and 2) monoubiquitinated PEX5 (stage 3 PEX5) which is completely resistant to the protease (see section 1.3.2.2 and 4.1) [76,78,83,84].

We first tried to detect PK-resistant species in *in vitro* import assays programmed with ^{35}S-PEX5(C11K)1-117 and ^{35}S-PEX5(C11K)1-125 using standard conditions (*i.e.*, incubation at 37 ºC in the presence of ATP followed by 30 minutes of proteolysis with 400 µg/mL of PK). Intriguingly, we were unable to detect protease-resistant species (data not shown).

We hypothesised that these negative results could be caused by the rapid export of these proteins from the DTM in the presence of ATP [84]. So, to increase the amount of PEX5 at the stage 2 level, we decreased the temperature of the *in vitro* assays to 26 ºC. As previously shown, under these conditions the ubiquitin-conjugating cascade acting on PEX5 is particularly slowed down [73].

As shown in Fig. 10, protease treatment of organelles containing ^{35}S-PEX5(C11K)1-324 (panel A, lane “+PK”) revealed the presence of stage 2 and stage 3 species, as expected. Note that the proteolysis conditions used in these assays are slightly different than the ones generally used in our laboratory [76]. Specifically, we decreased the incubation time with the protease given the problems in detecting protease-protected species for the small PEX5 proteins analysed in these assays. Thus,

instead of yielding a single species with the mass of the unmodified PEX5(C11K)1-324, some monoubiquitinated PEX5(C11K)1-324 remained intact after the protease incubation step. Unexpectedly, no protease-resistant species were detected in these experiments for ^{35}S-PEX5(C11K)1-125 (panel B) and ^{35}S-PEX5(C11K)1-117 (panel C). Apparently, DTM-embedded small N-terminal PEX5 fragments are accessible to the protease used in these experiments.

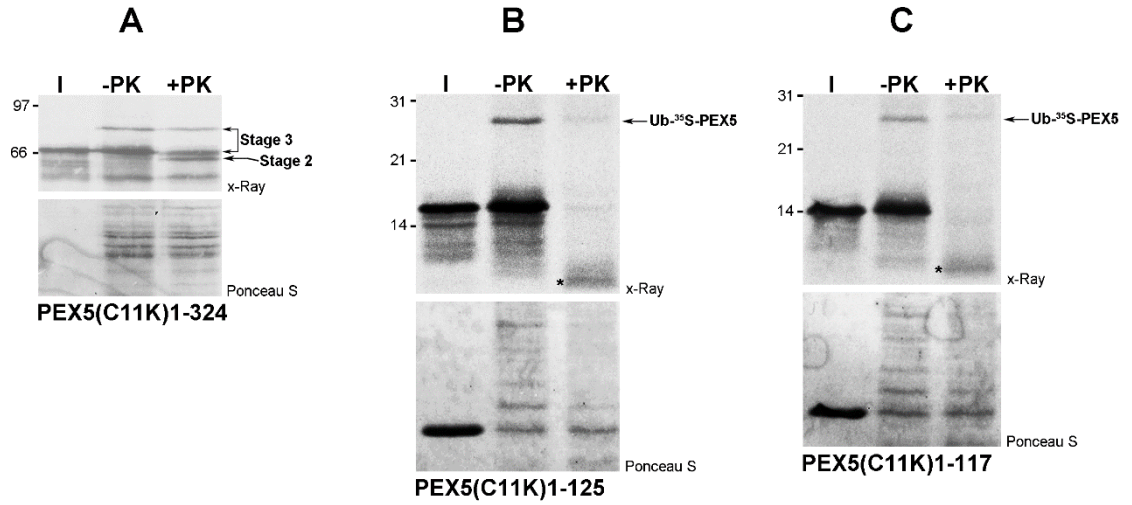

Fig. 10 - Protease-protection assay of ^{35}S-PEX5(C11K) C-terminal truncations.
Import reactions containing 600 µg of primed PNS protein were incubated for 30 minutes at 26 ºC with ATP and one of the following proteins ^{35}S-PEX5(C11K)1-324 (**A**), ^{35}S-PEX5(C11K)1-125 (**B**), ^{35}S-PEX5(C11K)1-117 (**C**). After import, reactions were subjected (**+PK**) or not (**-PK**) to a protease-protection assay with proteinase K for 15 minutes. Organelles were isolated and analysed by SDS-PAGE/autoradiography. **Lanes I**, represent 2.5% of the ^{35}S-protein used in each reaction. **Stage 2**, represents DTM-embedded non-ubiquitinated PEX5 from which a 2 kDa fragment is degraded by PK. **Stage 3**, represents DTM-embedded Ub-PEX5, here the PK does not cleave the radiolabelled protein but probably the ubiquitin moiety that is attached to the N-terminal of PEX5, yielding a species that migrates as full-length PEX5. **Ub-^{35}S-PEX5**, monoubiquitinated radiolabelled PEX5 species. *****, indicates radiolabelled products of the protease treatment that appear at the front of the gel irrespective of the ^{35}S-PEX5 protein used in the assay. Numbers on the left indicate the molecular mass of the protein standards in kDa.

To confirm these results, we repeated the protease-protection experiments with the C11A versions of some of the small N-terminal PEX5 fragments. The aim was to increase the amount of PEX5 proteins at the stage 2 level, and thus the sensitivity of our analysis (note that PEX5(C11A) proteins are not ubiquitinatable and thus accumulate massively at the DTM in *in vitro* assays supplemented with ATP; see section 4.1). As expected, ^{35}S-PEX5(C11A)1-324 can be easily detected at the stage 2 level in these assays (see Fig. 11, panel A, compare lanes “+PK” with “-PK”). We were also able to detected some PEX5 at the stage 2 level when using ^{35}S-PEX5(C11A)1-181 in these experiments. Intriguingly, two other proteinase K-resistant species were also detected (panel B, bands marked with “*****”). We do not know the origin of these bands but current work in our laboratory is being carried out to clarify this issue. The three other proteins used in these experiments, namely PEX5(C11A)1-158, PEX5(C11A)1-139 and

PEX5(C11A)1-125, yielded a negative result, that is, all were accessible to the protease. A possible explanation for this finding is provided in the discussion section.

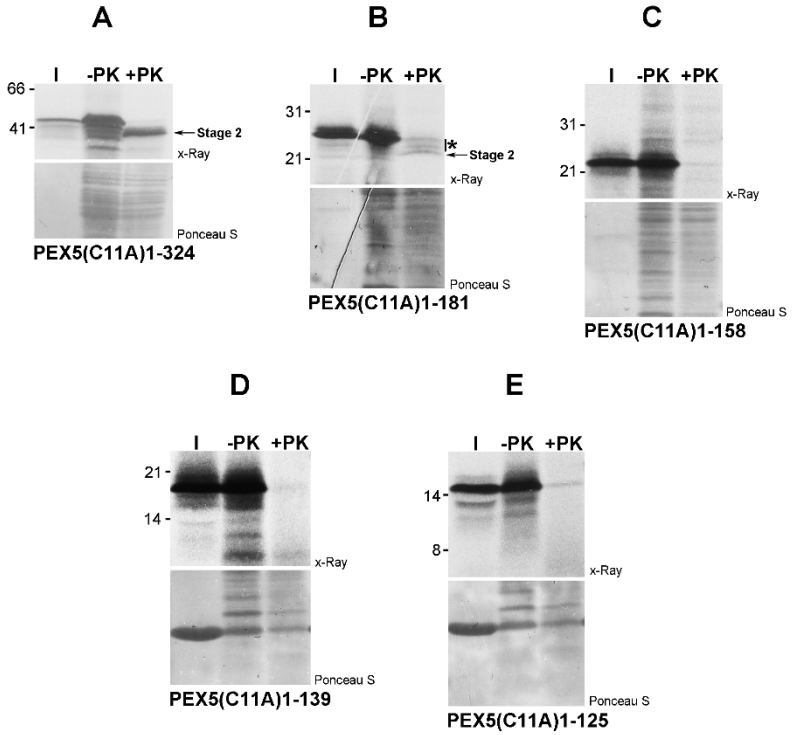

Fig. 11 - Protease-protection assay of PEX5(C11A) C-terminal deletion mutants.
Import reactions containing 600 µg of primed PNS protein were incubated with ATP and one of the following proteins ^{35}S-PEX5(C11A)1-324 (**A**), ^{35}S-PEX5(C11A)1-181 (**B**), ^{35}S-PEX5(C11A)1-158 (**C**), ^{35}S-PEX5(C11A)1-139 (**D**), ^{35}S-PEX5(C11A)1-125 (**E**). After import, reactions were subjected (**+PK**) or not **(-PK**) to a protease-protection assay with proteinase K for 30 minutes. Organelles were isolated and analysed by SDS-PAGE/autoradiography. **Lanes I**, represent 5% of the ^{35}S-protein used in each reaction. **Stage 2,** protease-resistant PEX5, indicated on the right; *****, protease-resistant radiolabelled proteins for which no explanation can yet be advanced. Numbers on the left indicate the molecular mass of the protein standards in kDa.

4.7 Recombinant PEX5(C11K)1-125 obtained in *E. coli* BL21(DE3)

As described above, both PEX5(C11K)1-125 and PEX5ΔN137 are import-competent. Remarkably, these two proteins are non-superimposable, meaning they correspond to two non-overlapping PEX5 regions. Since PEX5(C11K)1-125, in contrast to PEX5ΔN137, is export-competent, we wondered whether we could accumulate the two proteins at the DTM using our *in vitro* assay, and if so, whether the export of PEX5(C11K)1-125 might drive extraction of the PEX5ΔN137 protein. A positive result in such an experiment would reveal important mechanistic details on the mechanism used by the REM to extract PEX5 from the DTM. For instance, one might conclude that the REM acts on the DTM components, sequestering them from PEX5, and not on PEX5 itself; in such a scenario, ubiquitination of PEX5 would simply be the triggering signal to activate the REM and not a context-dependent handle for the export machinery.

To accomplish this aim, we needed to obtain large amounts of the PEX5(C11K)1-125 protein. We first generated a pET-28a derived plasmid containing a cDNA encoding the PEX5(C11K)1-125 protein, pET-28a-His_6-TEV-PEX5(C11K)1-125, which was transformed into *E. coli* BL21(DE3) cells. Although several experimental conditions were tested, we were unable to detect protein expression using this vector (data not shown).

We suspected that the inability to produce recombinant PEX5(C11K)1-125 in *E. coli* cells was due to its instability, probably derived from a combination of its small size and its natively unfolded structure. In an attempt to bypass this problem, we decided to express PEX5(C11K)1-125 as a fusion protein with SUMO3 (small ubiquitin-like modifier 3). This strategy improved significantly the amount of recombinant protein produced in these cells (see Fig. 12, panel A and B). However, the fusion protein was clearly very

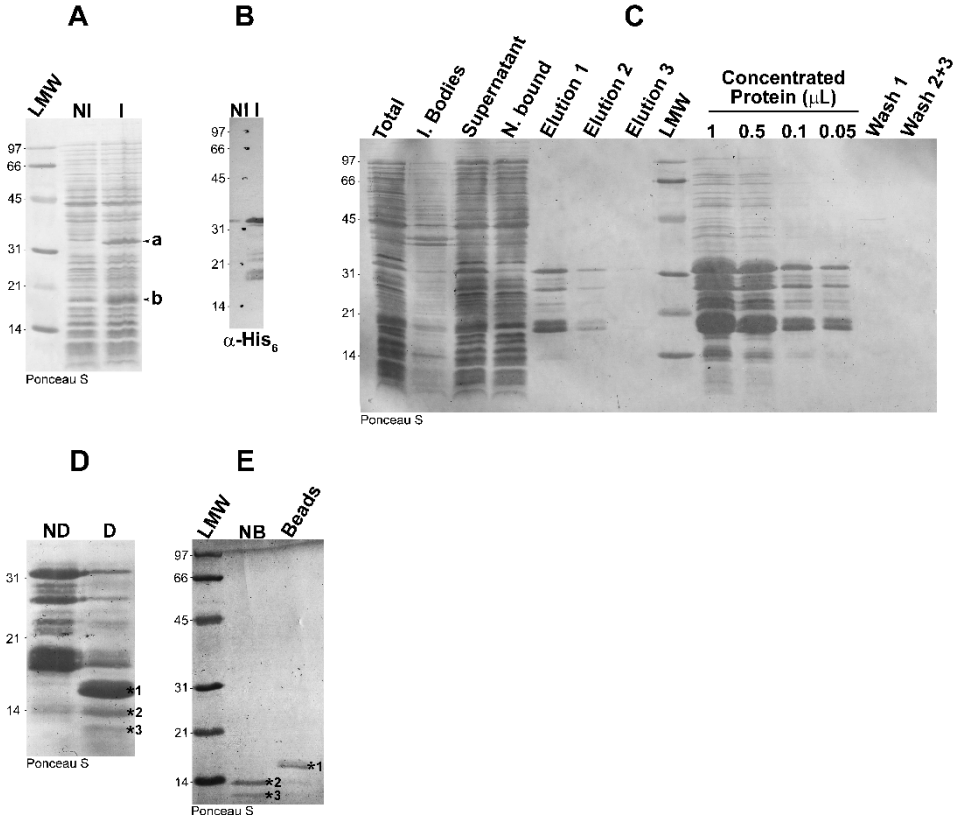

Fig. 12 - Production of recombinant PEX5(C11K)1-125.
A - Induction of the His_6-SUMO3-PEX5(C11K)1-125 protein expression was performed with 1 mM IPTG for 2 hours at 37 ºC. Aliquots equivalent to 100 µL of non-induced (**NI**) and induced (**I**) culture were analysed on SDS-PAGE; **a** and **b** indicate the induced proteins.
B - Anti-His immunoblot of 100 µL of non-induced (**NI**) and induced (**I**) cultures with 1 mM IPTG for 3 hours at 37 ºC.
C - Purification of His_6-SUMO3-PEX5(C11K)1-125 from *E. coli* cell extracts by Ni^{2+}-NTA affinity chromatography. Equivalents to 100 µL of culture were removed at different steps of the purification procedure, separated by SDS-PAGE, transferred onto a nitrocellulose membrane and stained with Ponceau S. **Total**, sonicated cells in lysis buffer; **I. bodies**, inclusion bodies recovered after centrifugation of the total sonicated cell lysate; **Supernatant**, soluble fraction of the total sonicated cell lysate; **N. bound**, non-bound fraction of the chromatography; **Elution**, aliquots removed after elution of the Ni-NTA beads; **LMW**, low molecular weight marker (BioRad); **Concentrated protein**, the final protein solution was successively diluted (as indicated); **Wash**, fractions resultant of washing the beads in buffer.
D - Digestion with cSENP2. 1.2 mg of His_6-SUMO3-PEX5(C11K)1-125 was cleaved with 1.2 µg of cSENP2 for 1 hour at 25 ºC. Aliquots equivalent to 2 µg of non-digested (**ND**) and digested (**D**) samples were run on SDS-PAGE, transferred onto a nitrocellulose membrane and stained with Ponceau S. The action of the SUMO protease gives rise to three new protein bands: *1, *2 and *3.
E - Cleaved His_6-SUMO3 was removed by Ni^{2+}-NTA chromatography (*1, **Beads**) and our protein of interest, PEX5(C11K)1-125, was recovered in the unbound fraction (*2 and *3, **NB**).
Numbers on the left indicate the molecular mass of the protein standards in kDa.

heterogeneous, the result of proteolysis acting on the PEX5 moiety (see Fig. 12, panel C, D and E). Furthermore, after cleaving the fusion protein with cSENP2 to remove the SUMO3 tag, we realized that the largest PEX5 fragment that we could detect was 1-2 kDa shorter than the ^{35}S-PEX5(C11K)1-125 protein obtained by IVT (see Fig. 13). Thus, we decided not to pursue these experiments further.

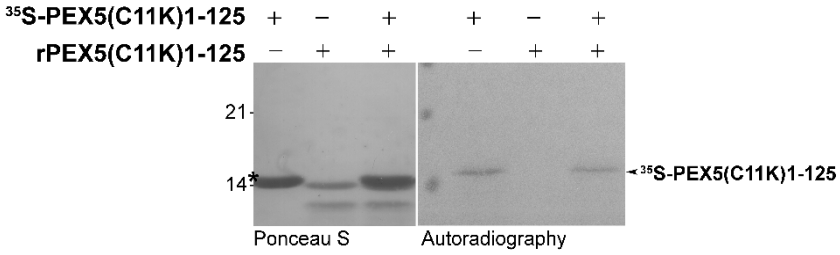

Fig. 13 - Recombinant PEX5(C11K)1-125 suffers proteolysis in *E. coli* BL21(DE3) cells.
Protein mixtures were analysed by SDS-PAGE and autoradiography. As indicated, either 1 µL of recombinant protein (which corresponds to *ca.* 0.7 µg of the 14 kDa protein) and/or 0.05 µL of ^{35}S-PEX5(C11K)1-125 were mixed and loaded. After staining with Ponceau S, the membrane was exposed overnight with an x-Ray film. The migration of the radioactive PEX5 is indicated with ◄. *, corresponds to the haemoglobin present in the rabbit reticulocyte lysate. Numbers on the left indicate the molecular mass of the protein standards in kDa.

5. DISCUSSION

Shuttling of the peroxisomal protein import receptor PEX5 between the cytosol and the organelle is an ATP hydrolysis requiring process [78]. Therefore, it is not surprising that the pathway is regulated and that only cargo-loaded PEX5 has access to the peroxisomal DTM [75]. As explained before (see Introduction section 1.4), several data suggest that this regulatory mechanism resides in PEX5 itself. All the presently available data indicate that the PEX5 C-terminal half is a *cis*-acting repressor of its N-terminal half (the region containing the DTM interacting motifs) but the molecular details behind this interaction remain unknown [75,116]. Our hypothesis is that the cargo-free C-terminal half of PEX5 interacts (and inactivates) the N-terminal half of PEX5, and that this interaction is disrupted when a cargo binds to the C-terminal domain of PEX5. We knew from previous work that the first 110 amino acids of PEX5 cannot be involved in this hypothetical cross-talk [73]. Indeed, as shown by Costa-Rodrigues *et al.*, PEX5ΔN110 is still import-competent in a cargo-dependent manner. In our work, we extended these studies by characterizing PEX5 proteins lacking larger N-terminal domains, namely PEX5ΔN137 and PEX5ΔN147. The results of this characterization are discussed below.

Our data show that PEX5ΔN137 is still able to insert into the DTM in a cargo-dependent manner. Thus, we can conclude that the first 137 amino acids of PEX5 are important neither for the insertion of the receptor into the DTM nor for its autoregulatory mechanism. This means that pentapeptide motifs 0 and 1 are required for none of these two processes. Rather different results were obtained for PEX5ΔN147. Indeed, this protein is no longer import-competent. Apparently, shortening PEX5ΔN137 by just 10 amino acid residues at its N-terminus completely abolishes its ability to interact with the DTM.

The fact that PEX5ΔN147 is no longer import-competent impedes us from drawing any conclusions regarding the cross-talk between the N- and C-terminal halves. Nevertheless, this property of PEX5ΔN147 might suggest that amino acid residues 138 to 147, where pentapeptide motif 2 is present, are precisely the N-terminal half region involved in the aforementioned cross-talk. Considering that pentapeptide 0 *per se* seems to be sufficient to target PEX5 to the DTM (see below), we could test our hypothesis by producing a full-length PEX5 protein having amino acid residues 138-147 substituted by alanines and determine whether this protein is import-competent but now in a cargo-independent manner.

The reason why PEX5ΔN147 is not import competent is intriguing; after all, this protein still contains pentapeptide motifs 3 to 7 which, in principle, might still sustain insertion of PEX5 into the DTM. However, as proposed before it is possible that the different pentapeptide motifs in PEX5 have different functions. For instance, some motifs

might be involved in the docking/insertion steps whereas others might be required for cargo-release [82]. In this scenario, our results indicate, on one hand that for the TPRs-containing PEX5 protein lacking pentapeptide motif 0 and 1 used in our study, pentapeptide motif 2 is crucial for the docking/insertion steps, and on the other hand that pentapeptide motifs 3-7 are unable to promote insertion of this PEX5 truncated protein into the DTM.

It is well established that the N-terminal half of PEX5 is crucial for its function as a shuttling receptor: it is through this region that PEX5 interact with all the components of the PIM, as shown by the finding that proteins such as PEX5(1-324) are fully competent in the docking, insertion, monoubiquitination and export steps whereas PEX5 proteins lacking the N-terminal half (*e.g.,* PEX5(324-639)) are completely incompetent at the very first step occurring at the peroxisome, *i.e.*, docking [unpublished,74,90].

In this work, we wanted to define the minimal domain of PEX5 functional in all of the steps occurring at the peroxisomal membrane. For this purpose, we produced several PEX5 C-terminally truncated proteins and characterized their behaviour in *in vitro* import/export assays. The results of these experiments and their implications are addressed below.

First, we found out that PEX5(C11K)1-117, a PEX5 truncated protein containing solely pentapeptide motif 0, still retains the capacity to enter the DTM and be monoubiquitinated. We also showed that a PEX5 protein without the TPRs domain and lacking the first 9 amino acid residues (*i.e.*, PEX5(C11K)10-324)) is still competent in the docking, insertion and monoubiquitination steps. Thus, these two observations indicate that the capacity to enter the DTM and be monoubiquitinated resides in the PEX5 domain comprising amino acid residues 10 to 117. We note that the monoubiquitination efficiency of PEX5(C11K)1-117 observed in our *in vitro* assays is lower than, for example, that detected with PEX5(C11K)1-324. This can be explained by the fact that human PEX5 contains several binding sites for PEX14, some of which are probably autonomous elements in the docking/insertion steps, whereas PEX5(C11K)1-117 contains only one PEX14 binding site, pentapeptide 0, with this capacity [77,113,114]. In other words, the existence of multiple PEX14 binding sites in PEX5 probably translates into faster association rates of this protein with the DTM because many more collisions between these two molecular entities will be productive.

Although PEX5(C11K)1-117 contains only one pentapeptide motif, its interaction with the DTM seems to be quite strong. Indeed, in the ubiquitination experiments showed (Fig. 7, panel D) no, or almost no, monoubiquitinated PEX5(C11K)1-117 can be detected

in the supernatant fraction of *in vitro* assays when AMP-PNP is used; only in the presence of ATP is this monoubiquitinated species exported into the soluble phase of the assays.

The mechanism used by the REM to extract PEX5 from the DTM remains largely unknown. From a conceptual point of view, there are two non-mutually exclusive possibilities (see Fig. 14) [127]. In one, the REM would bind Ub-PEX5 and would pull it from the DTM (panel A). In the other, the REM might disassemble the Ub-PEX5-DTM protein complex by interacting with and sequestering the membrane peroxins from the DTM that interact with PEX5 (panel B). Although our results are compatible with both models, the fact that monoubiquitinated PEX5(C11K)1-117 is exported in our assays has implications for the mechanism used by the REM to extract DTM-embedded PEX5 into the cytosol. For instance, it is now clear that the REM is not activated by some cargo-induced rearrangement of DTM components occurring when cargo-loaded PEX5 enters the DTM. Evidently, a PEX5 protein lacking any cargo-binding capacity can also activate and be extracted by the REM as long as it contains a ubiquitinatable residue at position 11 [75,89]. Also, our results suggest that amino acid residues 118-639 of PEX5 do not

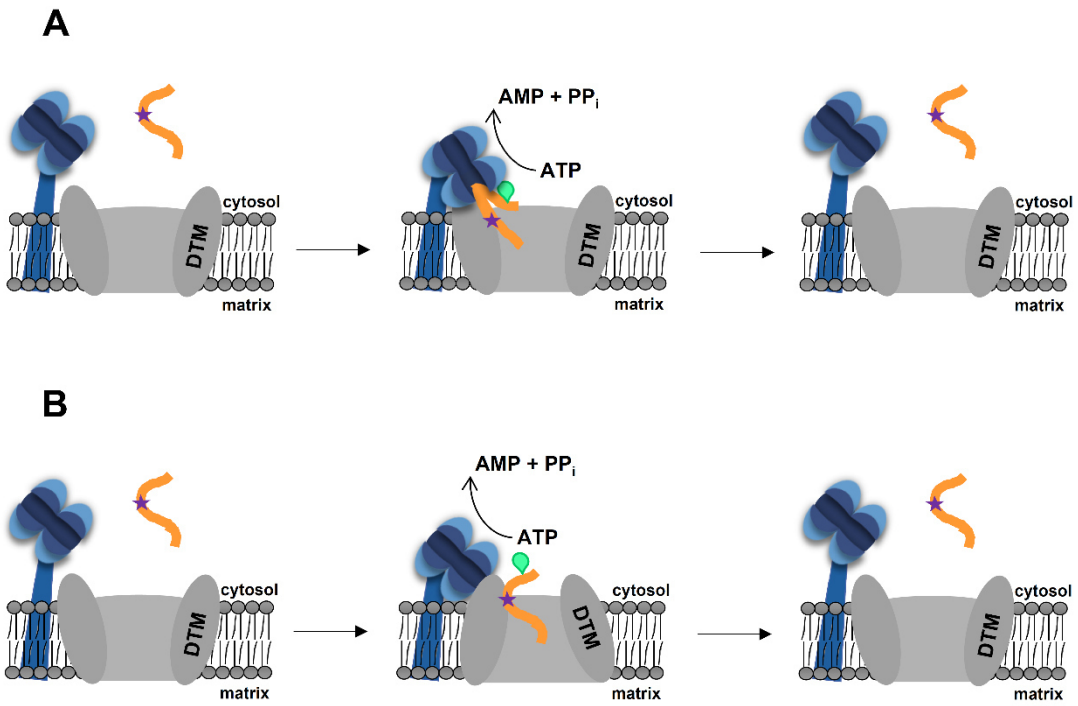

Fig. 14 - Possible mechanisms used by the REM to extract Ub-PEX5(C11K)1-117 from peroxisomal membranes. **A** - Ubiquitinated (bright green shape) PEX5 (orange line) is directly or indirectly recognized by the REM (blue complex). This recognition event possibly leads to an interaction between the REM and a loop of PEX5 contained in its first 117 amino acid residues. By coupling ATP hydrolysis to mechanical strength, the REM peroxins pull this PEX5 loop by a threading motion that ultimately results in Ub-PEX5 extraction. This mode of operation is similar to the AAA ATPase p97/VCP (Valosin-containing protein) which is responsible for the extraction of polyubiquitinated misfolded proteins from the endoplasmic reticulum [130]. **B** - Ubiquitinated (bright green shape) PEX5 (orange line) is directly or indirectly recognized by the REM (blue complex). This recognition event might lead to an interaction between the REM and the DTM (membrane-embedded grey complex). In this case, ATP hydrolysis is coupled to the mechanical strength exerted over the DTM, which leads to conformational alterations in the DTM that result in expulsion of Ub-PEX5 to the soluble fraction. This mode of action is related to the AAA ATPase NSF (N-ethylmaleimide sensitive factor) which disassembles the protein complex SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) after membrane fusion events [131].
The purple star represents the pentapeptide motif contained in PEX5(C11K)1-117.

establish crucial interactions with the REM. Apparently, at the very best only residues 10-117 of PEX5 are required for the export step.

The fact that PEX5(C11K)1-117 is monoubiquitinated and a substrate for the REM strongly suggests that this truncated version of PEX5 enters the DTM acquiring a normal topology. However, we were surprised by the finding that no protease-protected PEX5(C11K)1-117 was detected in our experiments. (As explained in detail in the introduction section, DTM-embedded PEX5 species are mostly resistant to proteinase K with the exception that 2 kDa of their N-terminus is removed by the protease, see Fig. 15, upper panel [75,78]). Moreover, exactly the same results were obtained for PEX5(C11A)1-125, PEX5(C11A)1-139 and PEX5(C11A)1-158 – none of these proteins displayed resistance to the protease in our *in vitro* assays. Although additional experiments must be performed to fully clarify these findings, there are two plausible explanations for our data (see Fig. 15, lower panel). One possibility is that cleavage of the cytosolic-exposed domains of the DTM components by proteinase K, weakens the architecture of the module, thus disrupting the interaction with PEX5(1-117) (see Fig. 15, lower panel, A) [77]. The other possibility is that proteinase K can enter the DTM whenever this membrane complex is occupied with the very small C-terminally truncated PEX5 proteins used in our study (see Fig. 15, lower panel, B). The latter possibility is

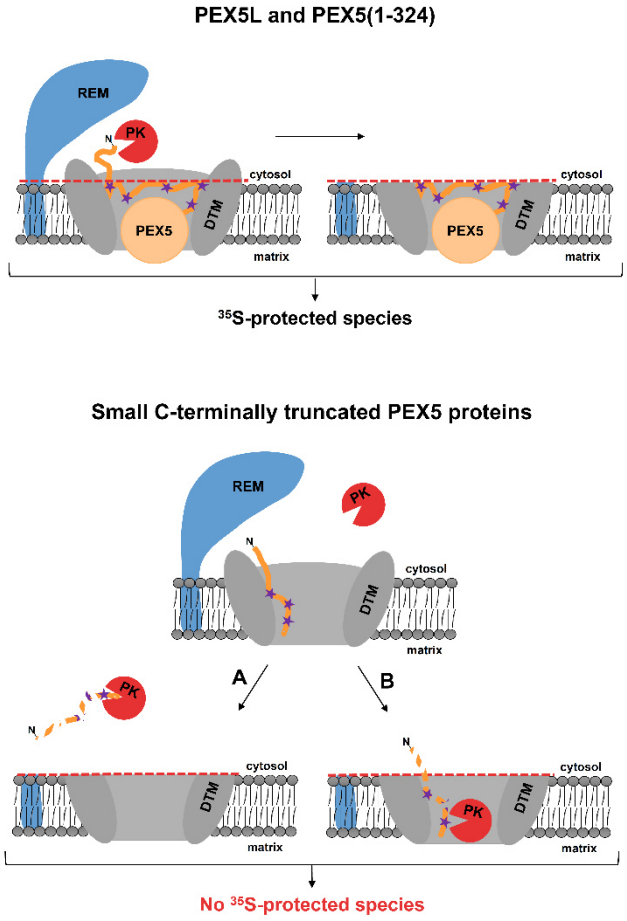

Fig. 15 - Molecular representation of a protease-protection assays after *in vitro* import of ^{35}S-PEX5 proteins.
Upper panel - *In vitro* imported full-length PEX5 or PEX5 amino acid residues 1-324 into intact peroxisomes, expose a small N-terminal fragment of *ca.* 2 kDa into the cytosol (in orange, the line represents the natively unfolded N-terminal domain and the sphere symbolises the TPRs domain). This region of PEX5 is susceptible to proteinase K (PK, in red) and is degraded. However, a significant part of PEX5 remains protease protected inside the DTM (grey membrane complex).
Lower panel - *In vitro* imported small C-terminally truncated PEX5 proteins (PEX5(1-117), PEX5(1-125), PEX5(1-139), PEX5(1-158), orange line) into intact peroxisomes, are totally susceptible to proteinase K (PK, in red), which can be explained by two possibilities. **A**, the protease degrades all the cytosol-exposed fragments of DTM peroxins. This disturbs the DTM stability and might lead to the disruption of the interaction between the small PEX5 proteins and the DTM. The PEX5 proteins now in the soluble fraction are degraded by PK. **B**, the protease enters in the DTM and degrades the small DTM-embedded PEX5 proteins. Note that proteinase K might enter because it is a globular protein with a radius of *ca.* 20.2 nm, whereas, for example, the PEX5 TPRs domain (one of the regions lacking in our small C-terminally PEX5 truncated proteins) can be approximated to a sphere with a radius of *ca.* 21.5 nm.
The purple stars represent the PEX5 pentapeptide motifs.

particularly interesting because it suggests that the DTM is a bowl-shaped cavity with a fixed geometry, which is fully filled by an intact PEX5 molecule, but only partially by the small PEX5 proteins used by us. In other words, the DTM would resemble more a mini-nuclear-pore(NPC)-complex and not the dynamic pore assembled on demand found in twin-arginine translocation (TAT) systems of bacteria and chloroplasts [128]. Future experiments need to be done to clarify this important aspect of the PIM.

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